The physiology of industrial yeast in continuous culture

Forbes Robert Wardrop B.Sc. (Hons)

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in collaboration with

Quest International Ltd.

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ABSTRACT

The physiology of industrial yeasts in continuous culture: F. R. Wardrop

The growth and physiology of *Saccharomyces cerevisiae* GB4918 (baker's yeast) was studied under glucose-limitation in chemostat culture. Levels of 1g/l (0.1% w/v) glucose allowed cell growth while preventing fermentation in a defined medium (QEMM3). Metabolism of glucose by respiration or fermentation was shown to affect the mean cell volume, with fermentative use of glucose causing an increase in cell size. This was also a major physiological difference between *S. cerevisiae* GB4918 (a Crabtree positive yeast) and *Kluyveromyces marxianus* DBVPG 6165 (a Crabtree negative yeast). The ability of the Crabtree positive yeast to substantially increase its' mean cell volume was also reflected in a 5-fold greater consumption of glucose, reduced biomass yield and increased ethanol yield, compared with the Crabtree negative *K. marxianus*. Growth of both these yeasts was seen in 50g/l glucose in the presence of the respiratory inhibitor, antimycin A. This was evident by the switching to fermentation in *K. marxianus*, and the complete fermentation of glucose by *S. cerevisiae*. The growth and physiology of *S. cerevisiae* GB4918 was also established in glucose-limited chemostat cultures, with special regard to the intracellular macromolecular compounds that are relevant to industrial yeast biomass production. This showed that in respiring cultures of *S. cerevisiae*, increasing growth rate resulted in decrease in both trehalose and glycogen content, while increasing protein and RNA. This is true until μ_{max} (in this context the growth rate at which respiro-fermentative metabolism occurs) when accumulation of trehalose and glycogen is apparent. Once μ_{crit} (growth rate at which washout of the culture begins) was reached then biomass significantly reduced. In describing the steady-state condition of baker's yeast it was then possible to describe changes occurring in yeast when subjected to a variety of nutrient perturbations. With a lactic acid (2% v/v) perturbation there were dramatic effects on both growth and metabolism at a growth rate of $0.12h^{-1}$, but significant decreases in biomass and protein, and significant increases in trehalose and glycogen. At a higher growth rate ($0.22h^{-1}$) the effect was much severer on protein content, and on reduced levels of trehalose and glycogen. The effect of perturbing the cultures with elevated levels of calcium was also most significant on reducing yeast trehalose and glycogen levels, probably due to inhibition of the biosynthesis of these compounds. Zinc additions to chemostat cultures acted to increase the levels of protein in the cells, while having little effect on any of the other cellular macromolecules. This suggests that increasing calcium levels during the latter stages of yeast propagations may produce a yeast with reduced stress responses. Increased zinc may also encourage a greater protein content, which would, in turn, provide a better nutritive content for both protein and amino acids in yeasts destined for use as a food additive.

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CHAPTER 1 - INTRODUCTION

1.1. Background to human exploitation of yeast

The micro-organism *Saccharomyces cerevisiae* is one of the most widely exploited biological entities utilised by humankind. It is also arguably one of the oldest, domestic species of any organism manipulated by man. Paradoxically, there have been very few successful attempts to isolate this yeast from the outside environment. There is a belief that it was, and still is, a common human commensal organism that was given its unique opportunity due to man's dietary requirement for a good carbohydrate source and the development of a "sweet tooth". This behaviour is constantly being back-dated by paleo-archaeologists, who have now discovered evidence that suggests that baking and brewing were practices of mankind before the established civilisations of Babylon, Sumeria and the Egyptians, who all have recorded the use of leavened bread and fermented beverages for general consumption and not just for the privileged classes as had been thought previously. Baker's and brewer's yeast have a long history in western civilisation and while both are species of *S. cerevisiae* much is argued about their relationship at the sub-species level. Historically, it would appear that the two were one and the same sub-species but as different brewing practices evolved then the yeast may also have evolved. Architectural plans for monasteries in the later centuries of the 1st millennium show that the process of brewing and baking were important in monastical life and that they were often housed together in the one building (AD part 2 400-800AD).

Since these early days of civilisation, advances in science and technology have allowed scientists of great historical distinction, for example Antonie van Leeuwenhoek's original description of yeast in the late 17th century, Louis Pasteur's work with yeast in beer (1876), and Emil Hansen's development of pure culture of yeast for brewing (1883), to identify and classify yeast species and highlight the importance of them in an industrial context. This is particularly true of Pasteur and Hansen both of whom are synonymous with brewing research. In more recent times the focus of attention has turned onto what else *S. cerevisiae*, and other yeast species, in general can do for humankind.

1.2. Yeast as a Food and as a Feedstock

The production of food from agricultural sources has now reached a major problem. Projections for both global population and food production show that both are rising. Significantly, human populations are growing twice as fast as food production. Using more land to produce more food is an obvious solution but this does not address some major hurdles which prevent this. The first of these is the need for land to house the increasing human population The second, which is related to the first, is the use of land for other purposes, i.e. industry, commerce and leisure. The final major concern is the growing understanding that large expanses of land have to be left uncultivated due to environmental concerns, or due to physical problems, e.g. mountains, lakes, rivers etc. Biotechnology is working to help solve this problem of land conflict by genetically improving crops in such a way as to increase yields of crop harvested from existing agricultural land.

However, there exist many alternatives to genetically engineering plants and releasing them into the environment. The best of all these is production of food and fodder products from micro-organisms. Already there are some microbial-derived food products available for human consumption, most notably 'Quorn' a protein rich fungal product produced through the growth of a species of *Fusarium graminearum* (Trinci 1994). This product is now widely available in Britain as a vegetarian alternative to meat, in products such as 'Quornburgers', that may be attractive to meat consumers who may be looking for a healthier alternative. 'Quorn' offers low fat as a major health benefit, but is also regarded as being a safer and more humane source of protein than meat such as beef, which in the UK has suffered greatly under the shadow of bovine spongiform encephalopathy (BSE).

One of the major problems with microbial-derived protein is that it does not, in many cases, offer the correct texture, and in the case of 'Quorn', egg material is used to help bind the pieces together to give it a more substantial mouthfeel. This is an important hurdle to cross but one mainly encountered in the West where there is less history of eating this type of foodstuff. Pacific states have used microbial protein in the form of algae throughout history as food and with recent technological advances are attempting to make this more economic on a larger scale. In the West our traditional micro-organism for food or fodder production has been yeast, mainly due to the large amounts of this organism left after brewing and wine making applications. Earlier this century brewers in Britain and Ireland made use of this waste as a food-stuff and produced 'Marmite' and similar products. As time has passed the understanding of the processes involved in yeast cell death has allowed yeast producers a more varied product

base than a simple yeast autolysate like 'Marmite'

However, the search for a micro-organism that could fully act as a non-meat, non-agricultural source of protein continues. Single Cell Protein (SCP) production is slowly being replaced by Single Cell Biomass as the major approach to provision of protein sources as the term SCP can only be applied to products that contain more than 65% protein (Boze *et al.* 1992).

S. cerevisiae belongs to a group of yeasts that are capable of both fermentative and respiratory metabolic use of carbohydrates, but are also subject to glucose-sensing mechanisms that forces the organism to preferentially ferment sugars when these are present above threshold levels. This is a characteristic of yeasts termed "Crabtree positive", where ethanol is produced at the expense of biomass, despite the adequate supply of oxygen for respiration to occur. Description of this behaviour is dealt with in more detail in section 1.6.1. The alternative to this type of yeast is a Crabtree negative yeast, i.e. *Candida utilis* which is also capable of both forms of metabolism, but only ferments when oxygen is unavailable. There are, in general, two other metabolic types of yeast, i) obligate (dominant) fermenters, e.g. *Candida pintolopesii* and ii) non-fermenting yeasts, e.g. *Rhodotorula rubra*. Boze *et al.* proposed that due to the predisposition of *S. cerevisiae* and other Crabtree positive yeast to fermentation that they were poor sources for food and fodder production. It is now regarded as a basic rule that attempting to produce food or fodder from yeasts should be done with a species that is Crabtree negative. It may be useful to avoid using strictly aerobic yeasts unless they offer some valuable advantage compared with Crabtree negative yeasts. The ability of *S. cerevisiae*

to ferment is exploited industrially during initial propagation steps where alcoholic fermentation is encouraged, as a safeguard against contaminants that may be present but also to provide a substrate (ethanol) that more effectively induces respiration than glucose.

Other major factors in selecting yeasts for biomass production are substrates and nutritional requirements. The more complex a nutrient source the yeast can grow on, generally, the better. It would be economic folly to select a yeast that required specific production of its nutrients rather than choose a yeast that can utilise substances found in some other form, especially industrial wastes, as with the case in the use of molasses as the major C-source in commercial production of *S. cerevisiae*. In terms of human nutrition, *S. cerevisiae* is an adequate source of many essential nutrients, including amino acids, trace elements and vitamins, especially those vitamins from the B-group. There is also the advantage that as a natural product it is more appealing as a dietary supplement than those that are currently available in health food outlets e.g. Zinc gluconate and Zinc picolinate. A typical analysis of nutrients available in yeast extracts is given in Table 1.1. This analysis is from *S. cerevisiae* and other yeasts may provide different nutritional profiles. This concept should also be kept in mind when considering a yeast extract for their flavour profile as not only will this change from yeast species to yeast species but altering the conditions under which the yeast was grown will also drastically effect the flavour profile of the subsequent extract.

Table 1.1 Composition of a typical yeast extract (from Nagodawithana 1995).

MAIN COMPONENTS (g/100g)		AMINO ACIDS (mg/g)	
Protein	67.9	Alanine	43.5
Fat	0.1	Arginine	32.0
Carbohydrates	17.6	Aspartic acid	64.1
Total sugar	4.3	Glutamic acid	106.6
Dietary fibre	4.14	Glycine	28.3
Moisture	4.00	Histidine	13.7
Ash	8.37	Isoleucine	30.1
		Cysteine	6.8
VITAMINS (mg/100g)		Leucine	43.7
Riboflavin	5.61	Lysine	49.8
Thiamine	7.36	Methionine	9.3
Niacin	7.68	Phenylalanine	26.4
Pantothenic acid	21.9	Proline	22.3
Biotin	0.05	Threonine	29.6
Pyridoxine	2.97	Serine	28.5
Folic acid	2.81	Tryptophan	6.38
Vitamin B_{12}	<0.001	Tyrosine	22.9
		Valine	33.8
MINERALS (mg/100g)			
Aluminium	5.96		
Barium	0.2		
Calcium	29.0		
Chromium	0.2		
Copper	0.12		
Iron	3.33		
Magnesium	40.2		
Manganese	0.15		
Phosphorous	1006.0		
Potassium	3520.0		
Sodium	815.0		
Zinc	17.4		

One of the other important factors concerning the future use of yeast in human and animal nutrition is the composition of the final extracts that can be used for these purposes. At present yeast extracts are the main way in which yeast is introduced into human diets (other than in bread and real ale) because the texture and mouth-feel of

yeast remains unpalatable to most consumers. Animal consumers, of course, are much less particular. With changes in attitudes towards health and continuing concern over food safety in most of the developed world, together with improvements in technology and understanding of downstream processing there may yet be a viable market for yeast products other than as additives to other products or as 'niche' health foods.

1.3. Flavours from yeast

As well as being a useful food source, yeast has also started to be considered as a food additive. Originally yeast was a nutritional source with some flavour characters but as technology has increased the ability to identify specific yeast-derived chemical flavour compounds has also increased. Two of the major flavour enhancers known in the food industry are monosodium glutamate (MSG), and inosine monophosphate (IMP) and both belong to substances termed "umami" (a term relating savoury mouthfeel). Both of these can be obtained from yeast, and their presence in yeast extracts makes the extract a more attractive additive than either chemical alone. The major character of these flavour enhancers is that they themselves have little or no aroma attributes but act in enhancing the activity of other molecules. These include other substances such as succinic acid and guanosine monophosphate, which, together with MSG and IMP, act to enhance flavours.

Meaty flavours are especially desired when considering applications such as soups and stock flavours. Public perception is a key factor in the use of substances as flavour enhancers. MSG is considered safe by the U.S. Food and Drug Administration (FDA) but has a negative image in the consumers mind especially with regard to

"Chinese Restaurant Syndrome", a set of adverse physiological reactions brought on by dehydration, due to the high levels of MSG used in food provided by these establishments (Ebert 1984). IMP, however, is a much more attractive product, but is expensive to produce as a pure chemical. IMP, and GMP are obtained from the enzymatic hydrolysis of RNA. Yeasts that are rich in RNA are valuable as food flavour enhancers with species such as *C. utilis* containing 10-15% RNA and baker's yeast normally containing 8-11% RNA (Stam *et al.* 1998). In order to maintain high levels of RNA when propagating yeasts growth rate must be kept high. However, at the end of conventional *S. cerevisiae* propagations there is normally a "ripening" phase which acts to reduce growth rate and, therefore, reduce cellular RNA.

Yeasts are also good sources of many other flavour compounds, some of which are outlined in Table 1.2. Important flavour compounds that are derived during yeast autolysis are the amino acids liberated from protein degradation. There are few examples in industry of yeast extracts with enriched levels of amino acids. Amino acids can provide bitter, sweet and sour flavours of varying intensity. An example of these are tryptophan as an intense bitter flavour that is not concentration dependent, and histidine as a less intense bitter flavour compound that is concentration dependent. There is a similar trend among the other amino acids, although a strange quirk is seen with the sweeteners that are produced from amino acid sources, some of which are not usually described as having sweet flavours e.g. Aspartame = aspartic acid (intense sour) and phenylalanine (slightly bitter).

Table 1.2. Major flavour compounds and their precursors in bread and beer produced by yeast fermentation (from Stam *et al.* 1998).

FLAVOUR MOLECULE	PRECURSOR MOLECULE	MAJOR SOURCE
Higher alcohols e.g. Phenylethanol, iso-amyl alcohol	Amino acids, e.g. phenylalanine, 2-ketoglutaric acid	Raw materials + amino acid biosynthesis
Organic acids	Sugars, fatty acids	Yeast 1° Metabolism, fatty acid biosynthesis.
Esters	Alcohols and acetyl CoA	Yeast 1° Metabolism
Carbonyl compounds: diacetyl, acetaldehyde	Amino acids	Raw Materials + amino acid biosynthesis
Sulphur compounds: DMS, H_2S, Cysteine, methionine	Amino acids, S-methylmethionine, dimethylsulphoxide	Raw materials, amino acid biosynthesis yeast autolysis
2-Acetyl-1-pyrroline	Amino acids, Glycolytic intermediates	Raw materials + Yeast 1° Metabolism
2-Acetyl-1,4,5,6,-tetrahydropyrdine	Amino acids, fructose	Raw materials + amino acid biosynthesis
3-Methylbutanol	Leucine	Raw materials + amino acid biosynthesis
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	Glycolytic intermediates	Yeast 1° Metabolism
2-Phenylethanol	Phenylalanine	Raw materials + amino acid biosynthesis
Phenylacetaldehyde	Phenylalanine	Raw materials + amino acid biosynthesis
3-Methylbutanal	Leucine	Raw materials + amino acid biosynthesis
Methylpropanal	Valine	Raw materials + amino acid biosynthesis
2,3,-butanedione	Pyruvate, acetyl CoA, monosaccharides	Yeast 1° Metabolism
Methional	Methionine	Raw materials + amino acid biosynthesis

1.4. Yeast Cultivation Techniques

Yeast (in this context, predominantly *S.* cerevisiae), due to its enormous industrial significance, has had many different culture handling techniques described for it and many have become major industrial practices in themselves. Like most micro-organisms, yeasts can be cultured on both liquid and solid media, and on interfaces presented by solid/liquid interfaces and air/liquid interfaces. However, the intention of this Introduction is only to describe the basics of the main culture techniques that are most frequently encountered in the yeast industry. Of the main techniques there are three (see Figure 1.1), and of these there exist many different varieties and, where appropriate, further distinction will be given to these variations.

1.4.1. Batch Culture

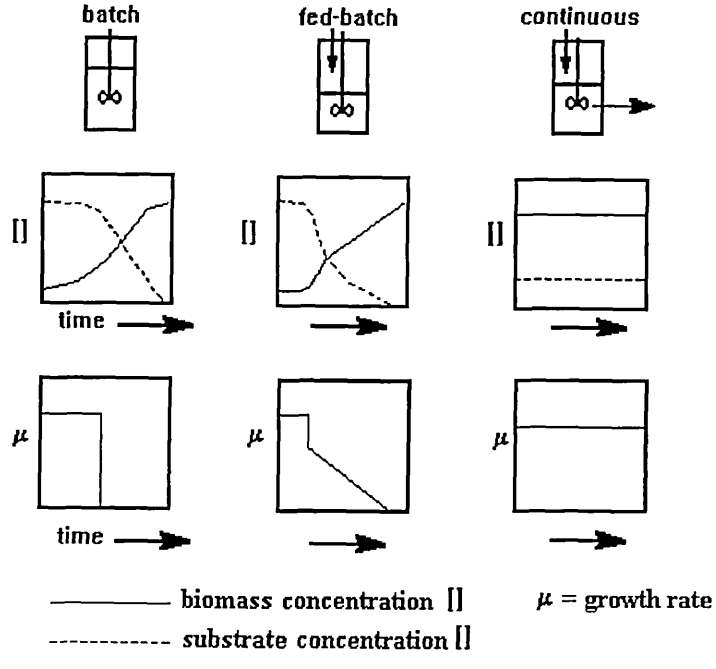
This is the simplest and easiest to use of all three yeast culture systems. It can be described as a system in which all necessary nutrients and conditions are prepared, the inoculum of yeast culture added and then left to its own devices until the particular process of interest has been completed. It is in batch culture that the typical growth phases of yeast are observed. These phases can vary in number, and six have been described by Fiechter *at al.* (1987) as follows. The first of these phases is **lag phase** or adaptation phase where the cells are apparently adapting to their new conditions and preparing for growth. This phase can last for a very long period of time and so reduction of lag phase is often best achieved by using exponentially growing cells as an inoculum. In general there is little, if any, increase in yeast biomass in this phase. The second phase is called **acceleration phase** where the cells have started to grow but are not yet doing

so in an exponential pattern. The third phase is **logarithmic** or **exponential phase** where the cells are growing at the maximal growth rate afforded by the conditions of their environment and nutrients available within. The fourth phase is called **deceleration phase** and is normally caused by lack of a key nutrient or in some cases accumulation of a toxic metabolite. The fifth phase is **stationary phase** and has recently started to attract more research into its precise physiology (Werner-Washburne *et al.* 1996). In this phase most cells adapt to the new conditions of nutrient starvation and can maintain themselves on stores for a while before limited cell lysis allows further maintenance of biomass levels. Eventually the sixth and final phase, the **decline phase** becomes predominant in which the majority of the population are undergoing the processes of death. Microbial cell death has been poorly understood until recent studies in *S. cerevisiae* have revealed important information with relevance to the processes of cell death in other cell types, including programmed cell death and apoptosis in mammalian cells and carcinogenesis (Shaham *et al.* 1998).

The use of batch systems is good for producing bulk quantities of a particular product from yeast cells (e.g. beer) but has many limitations. For example, it is difficult to ensure consistency of product produced within a batch system and it is very difficult to study the effects that various effectors have on yeast cells within such a system. Nutrient concentrations will be "unbalanced" and secondary metabolites may be produced meaning that the culture within a batch system is extremely dynamic with adaptations being made to changes in many parameters at any one time. Ultimately, in terms of biomass production, this sort of system is limited in the amount of biomass that can be produced from a given amount of substrate present at the beginning of the process. In

terms of biomass production, in Crabtree positive organisms like *S. cerevisiae*, batch culture is of little use as these organisms will begin to utilise excess carbohydrate (in most cases) in the production of metabolites and storage polymers rather than biomass. Other problems may also occur including the build up of extracellular metabolites which are inhibitory to growth (e.g. ethanol), and the difficulties of oxygen transfer as the biomass increases.

Figure 1.1 Diagrammatic representation of yeast culture systems



1.4.2. Fed Batch Culture

This system is a development from batch culture systems in that the inoculum is placed in the fermentation vessel with whatever nutrients it requires for growth supplied in a controlled manner, thus allowing the cells to develop as a normal culture. Using exponential phase cells as an inoculum enables the culture to get over any lag phase quickly and develop into an exponentially growing culture. At this stage "fed-batch process" begins and fresh supplies of required nutrients added. This can be done in one of two ways; the first involves a constant supply of medium at such a rate that the level of substrate (e.g. glucose or other sugar) is kept at low levels and results in an unstable specific growth rate; the second method involves maintaining the specific growth rate at a desireable level by altering the rate at which the feed medium is supplied.

The other advantages of the fed-batch system over the batch system include the fact that continuous feeding of nutrients allows the operator (and the yeast culture) to use much more substrate per unit time than is possible in a batch culture. Another advantage is that growth can be maintained for a longer period of time in the logarithmic phase and that the build-up of toxic metabolites is continually postponed through the dilution effect on the medium. However, as will be discussed later, the limitation of growing a yeast like *S. cerevisiae* is that oxygen transfer may become inefficient and biomass production becomes impaired as the yeast switches from a predominantly respiratory metabolism to a respiro-fermentative metabolism and eventually to predominantly fermentative metabolism.

In the industrial propagation of baker's yeast there are normally several propagation steps, most of which occur in a factory environment. The first propagation is normally carried out in a laboratory where sterility of the process can be easily maintained. This is done in 10-20L fermenters and will utilise either molasses or malt extract as a medium. It is paramount at this stage that the culture is pure, so as to prevent the possibility of a contaminant organism overrunning the subsequent industrial propagations. It is not yet economically feasible for industrial propagations to be carried out with sterile vessels or feedstocks. Equipment will be thoroughly cleaned, normally with a caustic solution, and then subjected to steam sterilisation which is normally effective enough. Medium components, especially molasses due to its viscosity, are very difficult to sterilise and so will only be subjected to a flash sterilisation. The molasses will be stored in steam sterilised vessels until required. The first stage of propagation in the factory will normally be carried out in a smaller vessel (500L) where the pure culture from the laboratory is inoculated into a molasses medium and allowed to ferment. This may seem like a wasteful procedure but it has more advantages to the yeast producer than disadvantages. This stage will allow three things to occur, 1) a limited amount of yeast growth, 2) a large accumulation of ethanol, 3) the subsequent re-assimilation of that ethanol with a concomitant increase in biomass. In two of these processes the aim of the producer is not being achieved, but the yeast is nevertheless adapting the environment in its favour. As previously discussed, the yeast producer attempts to maintain sterility as much as possible, but if there is a contaminant, present at very low levels, then the fermentative behaviour of the baker's yeast should facilitate monocultural growth. The third process, of distinct benefit to the yeast producer, is the aerobic utilisation of ethanol derived from yeast fermentation. This is allowed to continue until a

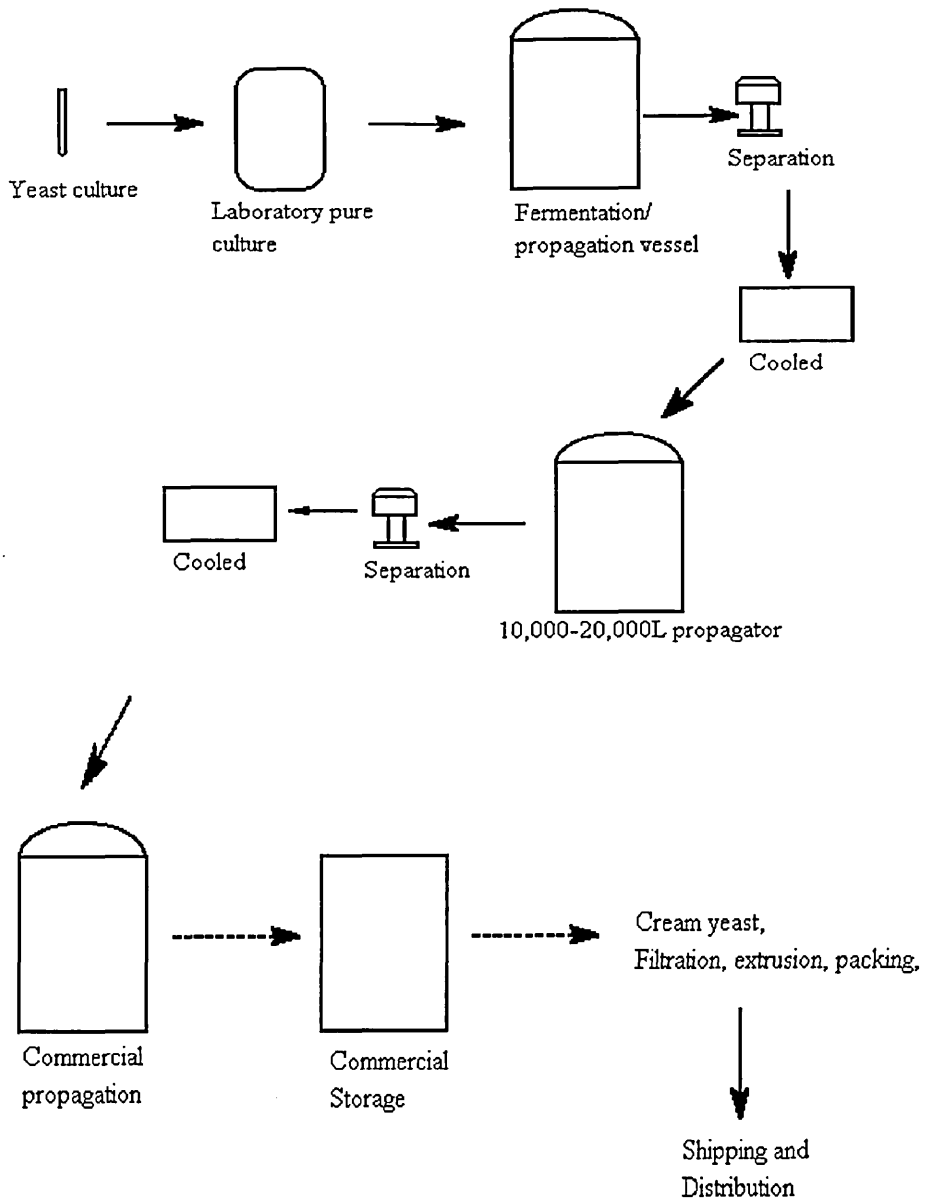
reasonable amount of biomass has been produced from the respiring cells. At this point the third propagative step will begin with the addition of feedstock (molasses and ammonium source) to the same vessel, normally at a slow feed rate. Once it is apparent that the cells are respiring, the molasses feed will be introduced in an exponential fashion, in order to maintain the exponential growth of the yeast. Once this stage is finalised (i.e. propagation vessel reaches its maximal volume), the yeast is separated from the spent medium and cooled before being stored in a cooled vessel. From here it will be inoculated into a larger propagation vessel (10,000-20,000L) where a further propagation step takes place. From here the yeast is again separated and cooled, stored in a cool storage vessel until it is required for the final propagation step. This step will see the yeast growing in vessels up to 200,000L, although 100,000L, is the usual size encountered (Kristiansen 1994). Yield of yeast at this stage can exceed 40 tonnes of wet yeast per propagation cycle, with output from a modest plant being in the region of 50,000 tonnes of wet yeast per year. This information is illustrated in Figure 1.2.

It is at this stage that the yeast can be dealt with in a variety of ways. Yeast for baking is often packaged as a cream into cooled tanks and transported in refrigerated tankers to commercial bakeries. Smaller bakeries and the home baker may be more used to 1kg blocks of pressed yeast, which is produced by rotary vacuum filtration and extrusion. This pressed yeast (about 30% dry matter) is stored in a chilled environment and has a shelf-life of one month, provided it is stored correctly. Storage at room temperature will increase the rate at which autolysis of the yeast occurs and result in a loss of leavening ability. Yeast may also be provided in smaller packages of active dried yeast, where the yeast is dried to about 93-94% dry matter. In this form the dried yeast

can survive for as long as two years, although 1 year is preferred. The yeast provided by this method has to be reconstituted in lukewarm water, in order to produce a yeast slurry with a reasonable viability. If the water used is too cool then the yeast cell membrane will take longer to rehydrate and this will result in essential components leaking out of the cells.

In the production of yeast for distillers, and for food additives it may be necessary for the yeast producer to introduce a further propagation step in which changes are introduced to the propagation medium in order to enhance a particular characteristic, e.g. the addition of more zinc to yeast that are bound for the distillers.

Figure 1.2 Diagram of the production of baker's yeast



1.4.2.1. Molasses

Molasses is the waste residue produced from the sugar refining industry and despite its description as a waste it still contains 40-50% w/v sugars. Sugar is obtained by mechanical and chemical extraction of plant sugars, mainly sucrose, from sugar beet or sugar cane. Sugar beet is a temperate crop grown in countries like Britain, Denmark and Poland and yields beet molasses. Sugar cane is the more abundant source of sugar crops and it is a tropical crop, grown in countries throughout the Caribbean and in South East Asia and yields blackstrap or cane molasses. A third type of molasses called high-test molasses, is produced by a method that increases the amount of invert sugars, and therefore reduces sucrose levels. Different extraction methods exist but even modern techniques are not effective at improving the yield of sugar from the sugar beet or cane without detrimentally effecting the quality of the molasses (Rosen 1987). Ion exchange methods are commonly employed in improving sugar extraction yields but this can lead to molasses with reduced Mg^{2+} levels (Rosen 1987) , which can provide a whole set of problems for the yeast producer. Before yeast production switched to molasses as its substrate, molasses had been, and is still primarily, used in low grade agricultural feedstocks and in the production of alcohol for Rum producers. Since the development of molasses as an industrial feedstock for *S. cerevisiae* many other applications have followed, mainly involving fermentative activity of yeasts and other micro-organisms some of which are related in Table 1.3.

TABLE 1.3. Fermentation products of Molasses

(adapted from Deshpande and Rale 1991).

Products	Organism
Rum	*Saccharomyces* sp.
Food:- Baker's yeast	*S. cerevisiae*
Vinegar	*Acetobacter* sp.
Feed:- SCP(protein supplement)	*Candida utilis, S. cerevisiae*
Probiotics	*Lactobacillus, Streptococcus lactis*
Organic acids:- Gluconic acid	*Aspergillus* sp.
Citric acid	*Aspergillus niger*
Lactic acid	*Lactobacillus* sp.
Acetic acid	*Acetobacter* sp.
Propionic acid	*Propionibacterium freundenrichii*
Gums:- Dextran	*Leuconostoc mesenteroides*
Pullulan	*Aureobasidium pullulans*
Solvents:- Acetone-butanol	*Clostridium acetobutylicum*
Ethanol	*S. cerevisiae*
Riboflavin	*Candida guillermondii, Eremothecium ashbyii*
Polyols	*Hansenula anomala*
Single cell oil	*Lipomyces lipofer, Rhodotorula gracilis, Rhodotorula glutinis*
Pullulans	*Aureobasidium pullulans*

The other major problem that yeast producers have with molasses is its consistency. It is an extremely viscous syrup, that has poor temperature conductivity, which makes sterilising it very difficult. In normal practice molasses feeds are not sterilised as such, but treated to a pasteurisation-type process. This not only saves energy in producing the heat required to sterilise it thoroughly, but also aids to prevent the reduction of its nutritional quality through condensation of the sugars with the amino acids in what are called Maillard reactions. These are caused by heating of the sugars in the presence of amino compounds, normally at neutral or alkaline pH. Low water concentrations also favour these "browning reactions" and result in compounds such as

hydroxymethylfurfural (HMF) dicarbonyls which are of no nutritional value to the yeast. However, these reactions are essential in providing the colour and flavour of bread crusts but can also be undesirable characters in powdered preparations of eggs and milk. Similarly, autoclaving of defined medium in which ammonium and glucose are present will also lead to browning of the medium which will correspond to a reduction in availability of both components to the yeast.

Further exploitation of agricultural wastes has now been elaborated upon in order for countries with their own peculiar crops to make use of the overcapacity in production that is becoming more prevalent as agricultural systems become more efficient. These can include plants like Jerusalem artichoke which researchers in France (Schorr-Galindo and Guiraud 1997; Schorr-Galindo *et al.* 1995) have focused on as this plant is a very rich source of the fructose polymer inulin. Alternatively a group in Algeria have begun to study how they can best make use of the 60,000tonnes/year of palm dates that are deemed unsuitable for human consumption (Nancib *et al.* 1997).

Table 1.4. Comparison of beet and cane molasses (from Rosen 1989).

	Beet	Cane
Dry matter (%)	74-78	75
Sugar - Total (%)	48-52	48-56
Invert (%)	0.2-1.2	15-20
Raffinose (%)	0.5-2.0	
Fermentable (%)	45-47	46-52
Unfermentable (%)		2-4
Organic non-sugars (%)	12-17	9-12
N-containing compounds (%)	6-8	2-3
Betain (%)	3-4	
Glutamic acid (%)	2-3	
Organic acids (%)	6-8	3
Gums, etc. (%)		4
Ash (%)	10-12	10-15
Na (%)	0.3-0.7	0.1-0.4
K (%)	2-7	1.5-5
Ca (%)	0.1-0.5	0.4-0.8
Cl (%)	0.5-1.5	0.7-3.0
P (%)	0.02-0.07	0.6-2.0
pH	7-9	5-6
Vitamins (ppm)		
Biotin	0.04-0.13	1.2-3.2
Inositol	6000-8000	6000
Pantothenic acid	50-100	54-64
Thiamin	1.3	1.8
Nicotinic acid	20-45	30-80

1.4.3. Continuous Culture

This method of yeast culturing is a development on the fed-batch theme except in this case while fresh nutrients are being continuously added, waste from the fermentation/propagation vessel is continuously removed. To call what is removed as waste is somewhat misleading, a better term is excess volume, as the general characteristic of continuous cultures is their constant volume. This is what makes continuous culture the best, in theory, of the three culture systems described here. Due to the constant nature of the continuous culture in can become a model system for studying the effects of one parameter on yeast/microbial growth kinetics and characteristics. It is possible to use what is called a chemostat, in which the chemical environment in the system has reached equilibrium , to study the effect of nutrients, the physical environment and even gene effects on a culture of cells. This can be more complicated in the other systems due to the dynamic physiological state of the cells growing in them and this dynamism makes it difficult to attribute any one change as being responsible for the overall character of a culture.

The chemostat is invaluable in research as time can be saved on optimising media for micro-organisms and also for identifying inhibitory constituents of natural medium sources. Excess volume is a better term than waste, due to the fact that what is removed from a chemostat in this manner is going to contain the product of interest, whether that is biomass or a metabolite or a recombinant protein. Of the three culture processes, continuous culture can provide the greatest productivity, with the duration of the process providing product, whereas both other culture methods require a complete halt in the process to obtain product.

Another major advantage of the continuous culture system is the ability to determine the effect of growth rate on the physiology of the organism in question or to study the effect of a single nutrient on the growth and physiology. The normal estimation of growth rate for cells in continuous, fed-batch, and batch cultures is carried out by determining the change in biomass per unit time (dx/dt). In batch cultures this growth rate (μ) is constantly changing, and can only reach μ_{max} during optimal nutrient supply during logarithmic stage of growth. In a chemostat, change in biomass depends not only on cell growth but on the rate at which cells are removed from the system. So in the chemostat, increase in biomass = growth - output; or $dx/dt = \mu x-(F/V)x$, where F is the flow rate and V is the volume of the fermenter and x = biomass. F/V = dilution rate, D, and therefore $dx/dt = \mu x-Dx$, D represents the dilution rate and is measured per hour (h^{-1}). This becomes one of the most important parameters of continuous culture since a steady state culture is described as one where there is no change in biomass ($dx/dt = 0$) in the vessel and thus $\mu x = Dx$ ($\mu = D$). If a microbial population within a chemostat remains well mixed and contamination-free then steady state may be achieved. Under such conditions, there is no change biomass levels, or in change of the substrates. At such a point it is possible to recover and study cells that are in a defined physiological state (Pirt 1975; Fiechter *et al.* 1987). Other characters that can be defined in chemostat culture include the maximum specific growth rate, which is both species and medium dependent. The same is true of the dilution rate that brings about the onset of respiro-fermentative metabolism in Crabtree positive yeasts. Growth is possible beyond this growth rate but the contribution of respiration is compromised by fermentation. Eventually a "critical dilution rate", D_c, is reached at which the culture is no longer capable of maintaining μ minus $D \geq 0$. Where this occurs the process of washout will

begin, in which the biomass is removed from the system.

An interesting phenomena that is often reported is the existence of oscillatory metabolism in yeast cells that are meant to be growing in steady state conditions. These oscillations manifest themselves in many metabolic parameters, ranging from dissolved oxygen, rate of carbon dioxide production and oxygen consumption, ethanol concentration, and intracellular carbohydrates (Satroutdinov *et al.* 1992). The mechanism that is responsible for these oscillations has still not fully been identified, but they appear to only occur at growth rates significantly lower than the optimal growth rate. One of the most favoured reasons for the introduction of these oscillations is based on the way in which the yeast cells are metabolising glucose. It has been reported that controlling the concentration of dissolved oxygen can eliminate oscillations, as can the addition of ethanol to the culture (Parulekar *et al.* 1986). This was further supported in work that showed increasing the flow of air into the chemostat could also eliminate oscillations and was more effective than increasing the concentration of dissolved oxygen (Keulers *et al.* 1996). This increased air flow rate suggested that a volatile compound may be the effector of the oscillatory metabolism. The influence of the cell cycle on these oscillation should not be dismissed since cell cycle-dependent oscillations have been demonstrated in chemostat culture (Satroutdinov *et al.* 1992; Duboc *et al.* 1996).

1.5. Physiology of *S. cerevisiae*

S. cerevisiae is described in taxonomic terms as belonging to the sub-family of the *Saccharomycetoidae*, which is part of the family of the *Saccharomyceteae*, in the order *Endomycetales*, which are found in the class of the *Hemiascomycetes*, in the sub-division *Ascomycotina*, in the division of the *Eumycota*, belonging to the Kingdom of the Fungi (Barnett *et al.* 1990). *S. cerevisiae* is described as being a unicellular organism, ellipsoidal in shape and generally reproduces by budding. There have been recent reports (Kron *et al.* 1994; Ramezani *et al.* 1998; Radcliffe *et al.* 1997) which have shown that *S. cerevisiae* does have the genetic ability to grow in a pseudohyphal fashion, although it is stressed that this is probably an adaptation to nutrient limiting conditions that allows the cells to seek more appropriate growth conditions (Maynard 1993).

The physiology of *S. cerevisiae* is a very complex subject area and for the purposes of this thesis will be kept to those areas that are most pertinent to the research undertaken. The yeast cells consists of many "compartments", ranging from the cell envelope (cell wall and membrane), to the cytoplasm, nuclear region, mitochondria, ribosomes, vacuoles, Golgi, endoplasmic reticulum, etc. Each of these compartments have important roles to play, and each has macromolecular components that are important to their functions. The nuclear region is the location of up to 90% of the yeast DNA, the remainder being located in the mitochondria, and small amounts in the cytoplasmic fraction. The mRNA that is transcribed in the nucleus is allowed to pass into the endoplasmic reticulum where ribosomes will translate the mRNA into proteins that will have functions throughout the cell. The cytoplasm is where the majority of the cellular activity takes place, especially in relation to metabolism, even more so when *S.*

cerevisiae is in its fermentative state. When respiring, the activity of the mitochondria will be at its maximal, with transport of metabolites between itself, the nucleus and the cytoplasm also at its most active. The regions that are of greatest importance to this thesis are the cytoplasm, mitochondria and the cell envelope.

1.5.1. Cell Wall

The yeast cell wall is the organism's first point of contact with its external environment. In general, cell walls in *S. cerevisiae* have been reported as having a thickness of around 25nm, in a cell whose overall diameter is 5-8μm (Bowen *et al.* 1992), although these dimensions will vary from species to species, and almost invariably will depend on the yeasts genetic background and growth. The cell wall may account for up to 25% of the total dry weight of the cell, but is normally in the region 15-25% of the cell dry weight (Rose 1993). The yeast cell wall is composed of polysaccharides (80-90%), proteins (10%), and small amounts of lipids, although some reports have suggested that the existence of lipids in the yeast cell wall is due to the presence of attached plasma membrane carried over in cell wall preparations (Rose 1993). The polysaccharides consist of a mixture of glucans, phosphomannans and small amounts of chitin. The glucans are largely responsible for the rigidity of the wall, especially the β(1-3)-linked glucans (Rose 1993). The chitin fraction is located in the bud scars and is generally believed to be closely associated with the β(1-3)-linked glucans. The role of the other glucan fractions is much less clear and includes the β(1-3)-linked glucan branched by some β(1-6)-linkages and the third fraction the β(1-6)-linked glucans.

It is the phosphomannan fraction of the cell wall that is much better understood, with the vast majority of this comprising mannans. The phosphomannans compose the majority of the outer layer of the yeast cell wall and it is the presence of phosphorus in phosphodiester linkages that are responsible for the overall net negative charge of the cell (Bowen *et al.* 1992). The other important aspects of the cell wall include the presence of enzymes such as invertase and acid phosphatase (Toda *et al.* 1982). The presence of acid phosphatase is well known in regard to the breaking down of the cell wall to allow bud formation (Linnemans *et al.* 1977). The role of invertase in the cell wall may not be consistent as the primary site of action of invertase on sucrose throughout yeast species, (Kaliterna *et al.* 1995).

The yeast cell wall is also the first line of defence for the yeast cell against its external environment and plays an important role in allowing molecules to pass into and out of the cell. This requires that the cell wall has a dynamic function, it is now known that the cell wall generally becomes thicker on entering stationary phase (Werner-Washburne *et al.* 1996). It is only very recently that the dynamism of the cell wall during the cell cycle has been elucidated (De Nobel *et al.* 1991) and this research showed how the cell wall varied in porosity throughout the cell cycle. The average pore size in the cell wall was 0.89nm, which is believed to prevent passage of molecules greater than 760kDa in size. The most porous stage of the cell wall coincided with the formation of the daughter bud cell which would arise due to the activity of the cell wall lytic enzymes that would be most active at this phase (Linnemans *et al.* 1977).

1.5.2. Cell Membrane

The yeast cell membrane is the more active environmental barrier, compared to the cell wall. It is composed mainly of polar lipids and proteins. The polar lipids are largely phospholipids and sterols, normally in a ratio of 4:1-6:1 (Rose 1993). There are two main phospholipids in yeast membranes, phosphatidylcholine and phosphatidylethanolamine, with minor phosphatidyl residues of serine, inositol and glycerol also present. The polar nature of the cell membrane serves as an effective barrier between the aqueous external and internal environments of the yeast cell. The sterol composition of the cell membrane is mainly ergosterol, ergosterol-residues and zymosterol. Protein is also a major component of the cell membrane as specific proteins are generally involved in transport mechanisms, including H^+-ATP-ase, an enzyme that is responsible for maintaining the proton motive force across the membrane. Other proteins of interest in the membrane include sugar transporters which are in a dynamic state depending on nutrient availability. The cell membrane is in a dynamic state during fermentation with cell membrane components being altered as the cell produces ethanol, which is a membrane active compound. If the plasma membrane remained a static structure, as in a non-ethanol environment, during fermentation, the ethanol the cell produces would severely effect the activity of the membrane and result in the death of the cell. However, during fermentation the cell alters the ratio of saturated to unsaturated fatty acids in the membrane, which allows the cells to withstand the activity of the ethanol that they are producing (Lloyd *et al.* 1993); (Swan and Watson 1997). There are other cellular mechanisms that prevent ethanol destroying the integrity of the plasma membrane, including trehalose which will be discussed later. Metal ions like zinc and magnesium are also known to act in the maintenance of the structural and functional

integrity of the plasma membrane (Dedyukhina *et al.* 1988; Walker *et al.* 1994a), but it would appear that this is a physico-chemical process rather than a cellular process.

1.5.3. Mitochondria

Mitochondria in *S. cerevisiae* have been shown to be physically and metabolically dynamic. The major role of the mitochondria is in generation of ATP for cellular anabolism, under aerobic conditions. During anaerobic or fermentation conditions mitochondria are less active, but not completely inactive (Visser *et al.* 1994). Mitochondria in aerobically grown yeast differ from those in anaerobically grown yeast (Visser *et al.* 1995). Mitochondria in yeast cells grown aerobically, either in ethanol or low concentrations of glucose, were more numerous and small ellipsoidal organelles, compared with those grown in anaerobic conditions which were fewer in number and with large, branched organelles. Visser and co-workers (Visser *et al.* 1995) were also able to determine that the change in mitochondrial morphology did not affect the fraction of the cellular volume occupied by these mitochondria and that these changes were a reaction to the repression of respiratory enzymes.

1.5.4. Cytoplasm

The cytoplasm is the source of glycolytic reactions in *S. cerevisiae* and is best described as a matrix. This description serves to explain that the cytoplasm is not devoid of structure and is itself a complex arrangement of molecules and not merely an "organic soup". Glycolytic enzymes are known to associate with the actin present in skeletal muscle (Clarke and Masters 1957 cited in Miyamoto *et al.* 1996), and activity of glycolytic enzymes was increased through association with actin. In yeast cells, this

activity could be improved through improving trehalose content, and subsequent improvement in gassing power of the yeast was coincident with increased levels of actin (Miyamoto *et al.* 1996). Cytoplasmic trehalose is a major focus of interest in this thesis.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), is a dimer of glucose with the glucose sub-units joined in a more unusual α1-1 glycosidic linkage, whereas sucrose and maltose use the chemically easier α1-4 glycosidic linkage. Trehalose is often referred to as a storage carbohydrate but while it is able to fulfil this role, it does not appear to be its primary function (van Laere 1989; Wiemken 1990). In fact, the ability of trehalose to take part in many of the cells' stress-related responses is of interest in the current research. One of the primary functions of a ripening stage in yeast production is to raise the levels of trehalose and glycogen in yeast that are bound for brewing and baking applications. However, the increased amount of trehalose in the yeast cell acts to protect it from some of the stresses that are involved in extracting yeast cells. Research has now shown that this remarkable disaccharide is capable of protecting a variety of structures in the yeast cell. For example, it can protect enzymes and proteins (Sola-Penna and Meyer-Fernandes 1994), as well as cell membranes (Iwahashi *et al.* 1995; Leslie *et al.* 1994; Mansure *et al.* 1994; Eleutherio *et al.* 1993). This wide activity of trehalose means that it may cause problems related to the processing of the yeast, if it is present at high concentrations. The ability of trehalose to protect many different cell types, not just yeast, is also well documented. For example, it is now used to protect tissues involved in surgical procedures (Kitahara *et al.* 1997). The reason why a relatively simple molecule like trehalose is able to do this is given by the number of equatorial hydroxide groups that is contains (Fujii *et al.* 1996). This is a function of

most sugars, but the more of these OH groups a sugar has the better it should be at protecting against both heat and pressure related stresses. It is now widely believed that trehalose acts to maintain structural integrity of the molecule it is protecting, probably by replacing water molecules that are being lost during the stress. During heat shock, which is the most studied stress mechanism, it is believed that trehalose plays a key role in protecting the cells, alongside other mechanisms including the heat shock proteins (Elliott *et al.* 1996). On its own trehalose is unable/ineffective at providing such protection. The metabolism of trehalose and glycogen in *S. cerevisiae* is shown in Figure 1.7. Both of these metabolic processes are subjected to many controls, one of which is environmental (heat shock). The enzymes involved in trehalose synthesis and degradation are normally always present, with the activity of catabolic or anabolic elements being controlled by glucose repression and growth phase of the cell.

1.5.5. Carbon Metabolism

The control of metabolism in yeast cells, and especially in yeast species like *S. cerevisiae* is extremely tight. The main areas of interest in the current research are the pathways involved in consumption of glucose and its utilisation as an energy source. This involves the major catabolic pathways in central metabolism, 1) glycolysis (Figure 1.3); 2) tricarboxylic acid (TCA) cycle (Figure 1.4); 3) electron transfer (Figure 1.5); 4) pyruvate dehydrogenase bypass (Figure 1.6); and 5) storage carbohydrates (Figure 1.7). These pathways account for the most likely fate of glucose in *S. cerevisiae* studied in this thesis and allow for a greater understanding of each of the metabolic processes.

Figure 1.3 The glycolytic pathway in yeasts, including glycerol production

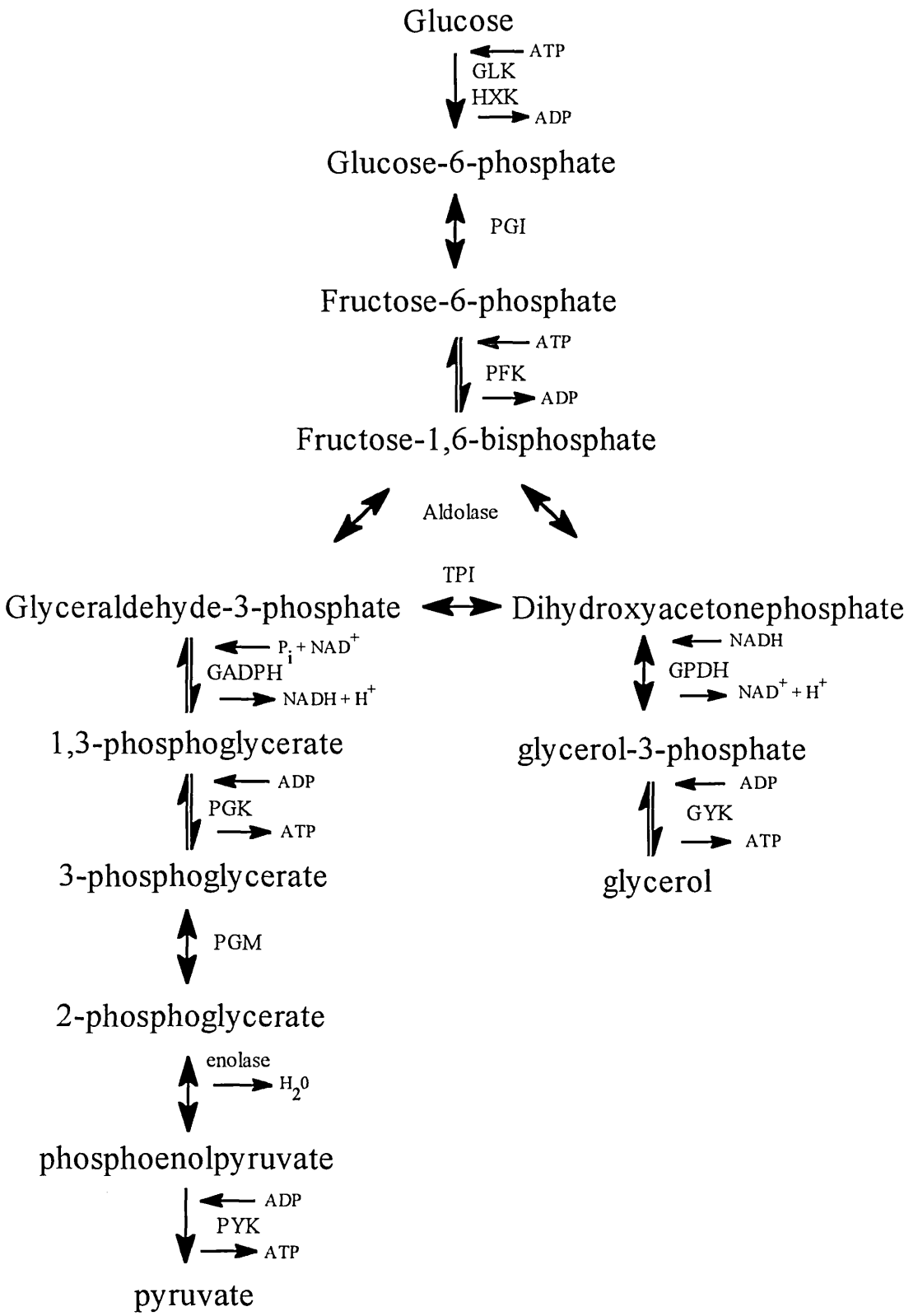


Figure 1.4 Tricarboxylic acid cycle

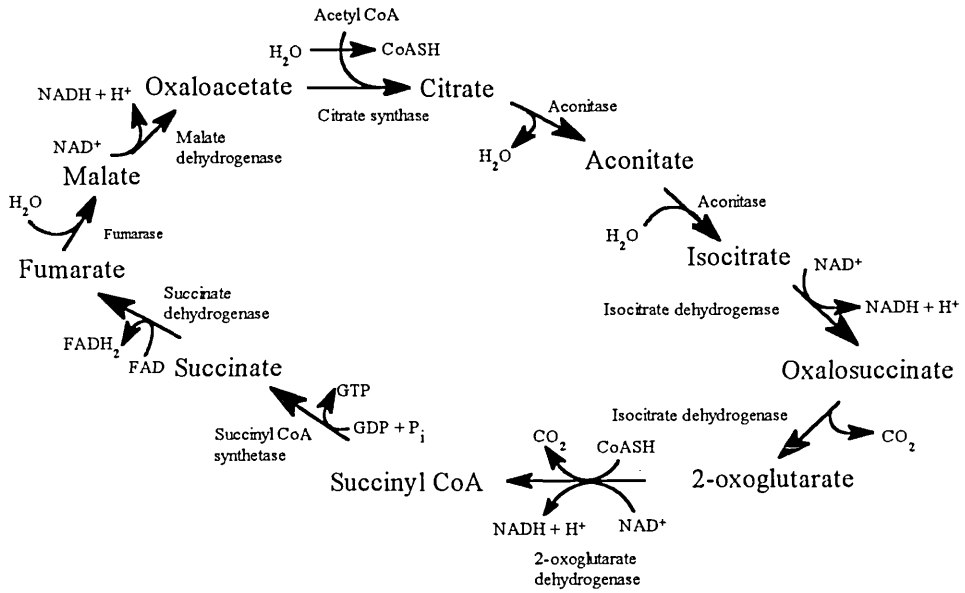


Figure 1.5 Electron transfer in *S. cerevisiae*

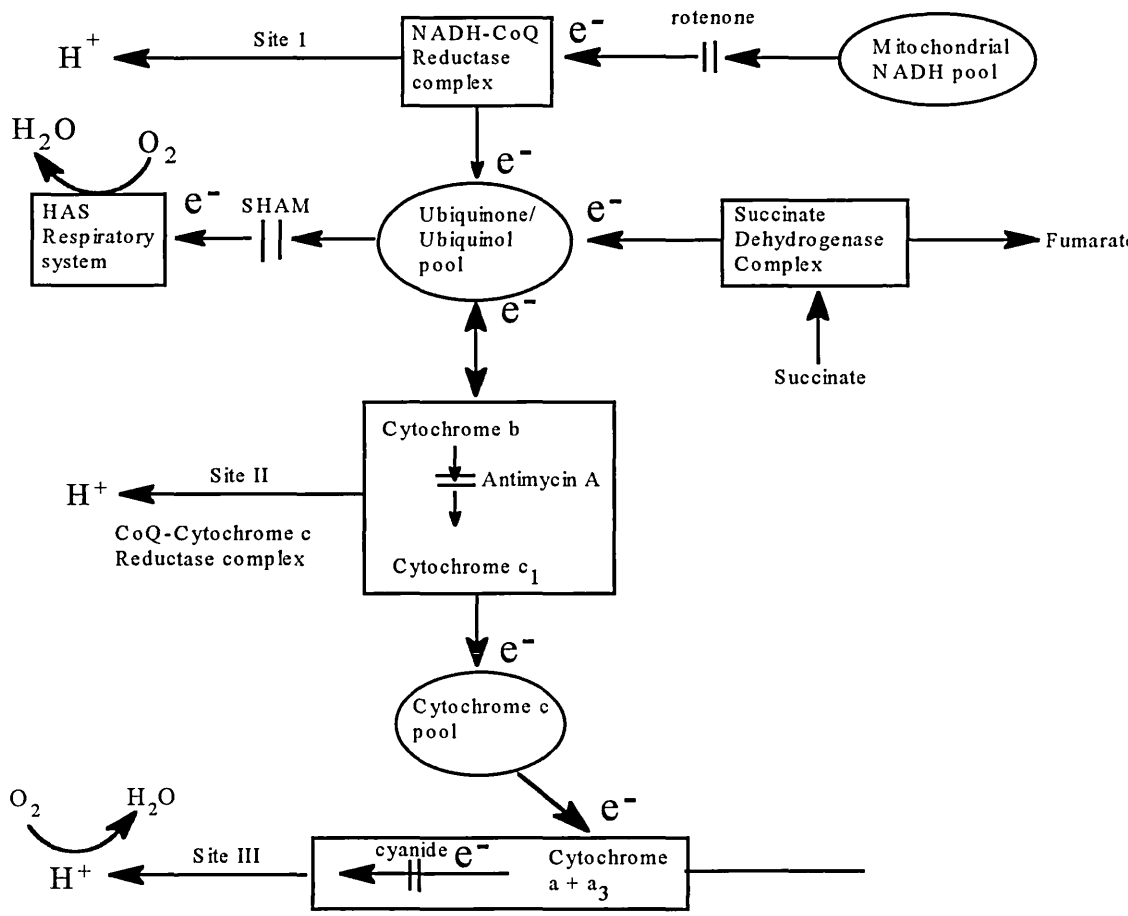


Figure 1.6. Pyruvate dehydrogenase bypass

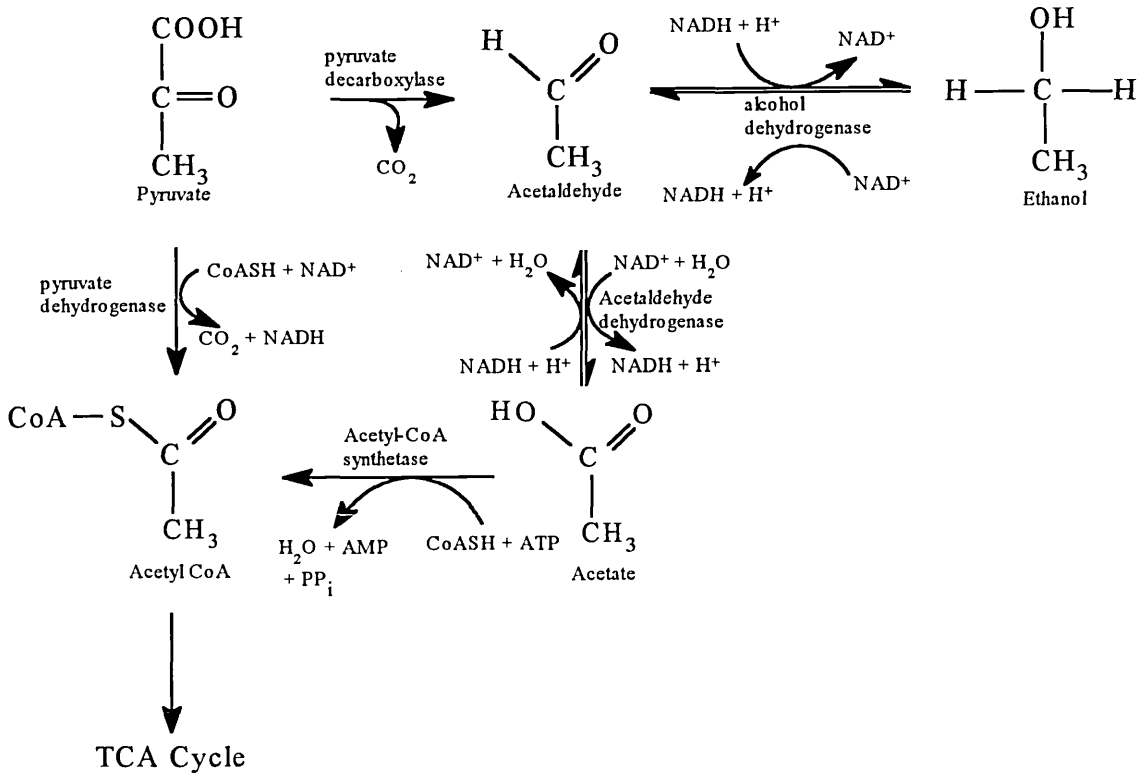
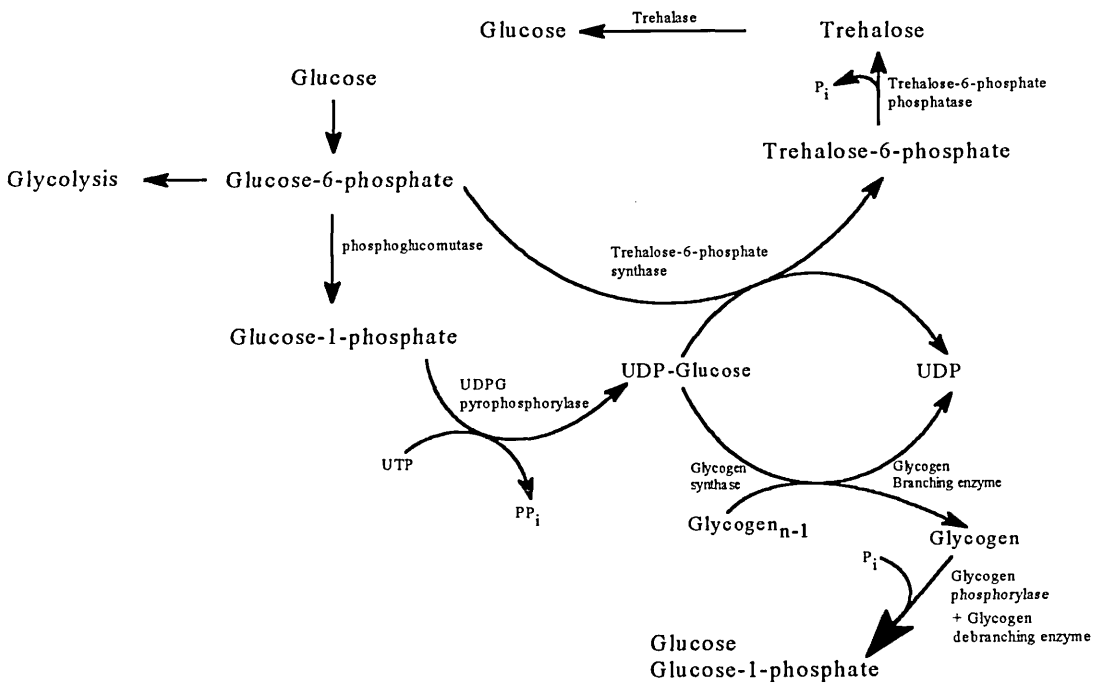


Figure 1.7. Metabolism of storage carbohydrates.



S. cerevisiae is capable of utilising a variety of sugars as carbon and energy supplies but glucose appears to be the preferred form of carbohydrate, closely followed by fructose. Both of these monosaccharides appear to be able to repress the utilisation of other saccharides, but the strongest repressive effect is observed with glucose. Sucrose is the main disaccharide of interest in this study due to being the principal saccharide in molasses. Sucrose utilisation requires the expression of the enzyme, invertase, and possibly the expression of a specific transporter (Mwesigye and Barford 1994).

Sucrose is a disaccharide that is composed of glucose and fructose monomers, joined in the 1-4 glycosidic linkage. Biologically, sucrose is digested by the enzyme invertase. Genes encoding for invertase and possibly a sucrose transporter are called *SUC* genes, and are among the most widely studied of the yeast genes. The existence of a sucrose transporter in *S. cerevisiae* is still a point of dispute. One has been described in *Debaryomyces yamade* (Kaliterna *et al.* 1995). While the presence of invertase is known in the cell wall (actually the periplasmic space), which requires passive transport of glucose and fructose into the cell, other research has suggested the existence of a specific sucrose transport mechanism (Mwesigye and Barford 1994; Mwesigye and Barford 1996). Sucrose utilisation is apparently controlled solely by glucose repression and is not subject to sucrose induction (Carlsen 1987). It has been shown that cells adapted to growth on sucrose appear to overcome the repressive effect of glucose to some extent (Mwesigye and Barford 1996).

It has been shown that yeast membrane transporters exist for maltose and trehalose, (Penalver *et al.* 1998; Crowe *et al.* 1991), both of which are repressed in the presence of glucose (Zimmerman and Entian 1998). The roles of a trehalose transporter would appear to be in the uptake of this sugar, but trehalose is not a widely found disaccharide, and the major role described for the trehalose transporter appears to be in allowing the disaccharide to act on both sides of the yeast membrane, when accumulated in the yeast cell (Eleutherio *et al.* 1993).

Maltose is the main sugar found in cereal-based media, such as brewer's wort and bread dough. Maltose is a disaccharide that is composed of two glucose monomers joined in an α1-4 glycosidic link and is digested by the yeast enzyme α-glucosidase, or maltase. Maltase and the maltose transporter are encoded for by *MAL* genes. These, as mentioned previously, are subject to glucose repression, but unlike the *SUC* genes they are also subject to maltose induction. Therefore, as glucose levels become lowered, maltose present in the medium can begin to induce the transcription of maltase and the maltose transport system (Zimmerman and Entian, 1998).

Galactose utilisation by yeast is dependent on a different assimilatory pathway altogether, known as the Leloir pathway. Whereas all the other sugars mentioned are merely hydrolysed and/or isomerised and phosphorylated to fructose-6-phosphate, galactose requires a separate pathway that allows it to enter glycolysis via glucose-1-phosphate. Galactose is similar to maltose, in that its pathway components, encoded by genes called *GAL*, are repressed by glucose. This is one of the best understood glucose repressed pathways, and is the reason why *GAL* gene regulatory regions are often used in

genetic transformation studies. The *GAL* genes are strongly repressed by glucose and induced by galactose, which is evident from the 1000-fold increase in mRNA from *GAL* genes when galactose is present as a promoter (Zimmerman and Entian 1998).

S. cerevisiae is also capable of using many other carbon sources, several of which can be produced by the yeast itself during its metabolism. These include some pentose sugars, (e.g. xylulose and arabinose); alcohols (e.g. ethanol); polyols (e.g. glycerol); and organic acids (e.g. citrate, succinate, acetate). Some of these compounds can only be utilised respiratively, e.g. acetate, ethanol, and glycerol, and most are subject to repression by glucose.

1.5.6. Oxygen

After carbon metabolism, oxygen is the next nutrient source of interest due to its role in metabolic effects such as the Crabtree and Pasteur effects (to be discussed later). Oxygen is an important factor in the physiology of any yeast that is not an obligate anaerobe. In all the other metabolic classifications of yeast oxygen is required either as an electron acceptor at the end of the respiratory chain or as a component in sterol synthesis. In the case of a yeast like *S. cerevisiae*, oxygen is an essential requirement in both of these situations. In fact, it is this requirement for oxygen in sterol synthesis that is one of the characteristics that has disqualified *S. cerevisiae* as a facultative anaerobe (Lagunas 1981). Practical evidence for this is the practice of sparging air or oxygen into brewery wort before or at the start of the brewing process. Some research has shown that yeasts with impaired membrane function when pitched into brewer's wort have difficulty in utilising available sugars in the wort (Boulton and Quain, 1987). However,

it has also been stressed that the levels of oxygen in brewer's wort must be closely controlled, because too high an oxygen level will almost certainly affect the characteristics of the beer (Kirsop, 1974). One characteristic that is of paramount importance in brewing is the minimal production of biomass during fermentation, which is contrary to the aim of yeast producers. However, biomass levels are believed to be unaffected until the respiratory quotient of the yeast falls below 10 (Kuriyama and Kobayashi 1993). The respiratory quotient increases due to increases in dissolved CO_2 with ethanol yield increasing at the apparent expense of glycerol production (Kuriyama *et al.* 1993).

1.5.7. Nitrogen

Yeasts in general are able to utilise a wide variety of nitrogen sources, but not molecular nitrogen. Nitrogen can account for up to 10% of the yeast dry cell weight (Jones *et al.* 1981). Ammonium sulphate is often used in defined medium as an N-source and ammonium hydroxide is used as a molasses supplement in commercial yeast propagations. The use of ammonium requires the increased use of cellular energy and carbon sources as building blocks for amino acid synthesis, which are in turn essential for protein synthesis. This has the effect of lowering biomass yield in aerobic propagations and reducing ethanol productivity in fermentations (Jones *et al.* 1981). *S. cerevisiae* is also able to utilise free amino acids and small polypeptides as N-sources. This would help the yeast cells produce biomass/ethanol more efficiently than using ammonium sources, but the cost of protein hydrolysates is more than that of cheap agricultural grade, ammonium sulphate or ammonium hydroxide.

1.5.8. Phosphorus and Sulphate

Phosphorus is required by yeast for DNA/RNA biosynthesis and in energy metabolism as well as being present in phospholipids. Phosphate units are largely responsible for the net negative charge of the yeast cell envelope. Phosphate is used by the yeast cells normally in its orthophosphate form (H_2PO_4), and is generally stored in the yeast vacuole (Okorokov *et al.* 1980). Sulphur is only required for sulphur containing amino acids, and the preferred source is methionine. This is an expensive supplement for industrial-scale yeast growth so ammonium sulphate is used to supply the required sulphate. However, this has the effect of reducing cell yields as SO_4 has to be metabolised to aspartate before the cells can produce methionine.

1.5.9. The role of metal ions

Yeast have a requirement for many metal ions, although both potassium and magnesium are required in greater concentration than the majority of the others such as zinc, manganese and iron. Most of the need for these elements is in the function of enzymes. In particular, magnesium is known to be required in over 300 enzymes and is strictly required in phosphate transfer systems, especially those involved with ATP (Walker 1994), as can be seen from Figure 1.2, this includes the majority of glycolytic enzymes. Glucose-6-phosphate is produced through the activity of hexokinase and glucokinase. Both enzymes require magnesium, as do phosphofructokinase, phosphoglycerate kinase, enolase and pyruvate kinase. Several of the TCA cycle enzymes also require magnesium as a co-factor. Yeast appears to tightly regulate cellular levels of magnesium (Beeler *et al.* 1997) and calcium (Maguire 1995). Potassium is also absolutely required for yeast growth. It has many roles as a co-factor

in oxidative phosphorylation reactions, and as a general charge balancer. Potassium ions are linked to the uptake of both magnesium and phosphate (Jones and Greenfield 1984; Wacker 1968).

Calcium is required in lower concentrations (generally micromolar, as opposed to millimolar requirements for magnesium and potassium) than magnesium and most of its biological activity in yeast cells appears to be related to cytoskeletal function and in signal transduction cascades and in the process of flocculation (Rose 1993). The other reported phenomenon with calcium in yeast cells is its antagonistic relationship with magnesium (Maiorella *et al.* 1984; Saltukoglu and Slaughter 1983; Walker *et al.* 1996; Iseri and French 1984; Walker 1999). The active exclusion of calcium by yeast cells appears to be part of the regulation of cellular magnesium content (Walker 1994; Beeler *et al.* 1997).

The final metal ion of interest with regard to current research is zinc, which is also absolutely required for yeast growth. Zinc plays the role of a structural stabiliser in some proteins, and is involved in the DNA replication machinery. It is also required in the synthesis of riboflavin as well as being an important catalytic centre in the enzymes alcohol dehydrogenase and acetaldehyde dehydrogenase, both of which are essential in yeast growth and fermentation. Growth of yeast in the absence of zinc has been shown to be impossible and in zinc deficient yeast differential expression of several proteins is known to occur (Obata *et al.* 1996). Zinc is also quite unique among metal ion requirements in that no other ion has yet been reported to be able to replace it in its functions.

There are many other metal ion requirements in yeast, including cobalt, copper, iron, manganese, and nickel (Jones and Greenfield 1984). Most of these elements have unspecified activities although improved growth or fermentation is normally described when they are present at optimal levels. The exception here is iron which is essential for the activity of the haem-enzymes involved in electron transfer. Other metal ions such as sodium, lithium and aluminium, are all described as having only deleterious effects on yeast growth (Jones and Greenfield 1984).

1.6. Control of Metabolism

All of the pathways detailed in Figures 1.3-1.7 are controlled by mechanisms in order to prevent build-up of toxic intermediates, such as trehalose-6-phosphate, glucose-6-phosphate and acetaldehyde. In addition, where reverse reactions take pyruvate to glucose in the gluconeogenic pathway, there must be control exerted to prevent a futile cycle of glucose-pyruvate-glucose, which would prevent the cells from growing.

The major regulatory mechanism in yeast carbon metabolism is the extremely complex process of glucose repression, or carbon catabolite repression (Gancedo 1992). In *S. cerevisiae*, this serves to maximise the utilisation of glucose first before other carbohydrates can be utilised. The major pathways that are repressed by glucose are: i) gluconeogenesis, preventing the futile cycling mentioned above, ii) mitochondrial enzymes involved in TCA cycle and electron transfer, and iii) uptake mechanisms for other carbohydrate sources that may be present, e.g. sucrose, maltose and galactose (Trumbly 1992). It would appear that these three systems are relieved in reverse order, so that if the next carbon source is fermentable then it is fermented. Once these sources

are exhausted, the cell will then turn to respiratory sources such as ethanol, and therefore mitochondrial enzymes are glucose de-repressed. Finally, once all external sources are exhausted it may be necessary for the cell to begin synthesising its own glucose for the purpose of maintaining reserves of glycogen and trehalose and other metabolic intermediates that may be required for anabolic processes. Trehalose biosynthesis is now also believed to play an important role in the regulation of glycolysis (Thevelein and Hohmann 1995). This has been shown by the inability of yeast strains to accumulate trehalose. In particular those that have a mutation or deletion of the *TPS1* gene, which encodes the trehalose-6-phosphate synthase enzyme. These mutants, *Δtps1*, are unable to grow on glucose or fructose, and have been identified as possessing the same mutation present in a gene believed to code for a general glucose sensor (*GGS* gene). More recent studies have shown that growth can be re-established by causing mutations that reduce sugar uptake (Luyten *et al.* 1993), e.g. Δ*hxk2*, hexokinase 2 gene (Thevelein 1996).

Carbon catabolite repression effects both DNA transcription, e.g. repression of *SUC*, *MAL*, and *GAL* genes, and the stability of mRNA, which is degraded quickly. It has been reported that a balance of both of these effects may occur on some enzymes, as shown with the iron protein subunit of succinate dehydrogenase (Cereghino and Scheffler 1996). Carbon catabolite repression is one of the major controlling factors in the initiation of the Crabtree effect in batch cultures of *S. cerevisiae*, and hence, is the reason why fermentation dominates over respiration under conditions of abundant sugar and non-limiting oxygen supply. Catabolite inactivation is another method of metabolic control that works in an immediate fashion to deactivate enzymes involved in yeast sugar metabolism.

1.6.1. The Crabtree effect

This is the metabolic phenomenon described by Crabtree in 1928, an observation that he made in tumour cells. The effect is described as the preferential fermentation of glucose/sugar, even in the presence of oxygen. This has proved misleading due to the effect of catabolite repression discussed above, especially in batch cultures. Continuous culture has allowed investigations into the nature of the Crabtree effect and has shown the existence of both long-term and short-term Crabtree effects. The former arises when the cells are adapted to the conditions and still continue to preferentially ferment glucose, but still carry out a background respiration. This was deemed to be due to a "limited respiratory capacity" (Kappeli and Sonnleitner 1986) that may be common to most Crabtree positive yeasts. Short-term Crabtree effects also exist where a non-fermenting yeast culture suddenly ferments when supplied with excess sugar. This short-term effect has been hypothesised as a saturation of respiration at the pyruvate branchpoint in metabolism (Pronk *et al.* 1996). This has also been shown to be effected by the levels of the enzyme pyruvate decarboxylase (Pdc) present in the cell (Van Hoek *et al.* 1998a). When Pdc was overexpressed in glucose-limited continuous cultures, cells showed a lower growth rate at which the Crabtree effect occurred. The Crabtree effect is inducible in continuous culture, without increasing glucose concentration. This is seen as evidence for the purest expression of the Crabtree effect being due to a limited respiratory capacity, as no glucose increase is involved. It should be noted, however, that glucose itself cannot be the sole effector of the effect, as free glucose is not found within yeast cells. Therefore, another metabolite or cell signal must be responsible for the effect of glucose repression. Fructose is also preferentially fermented under these conditions, but mannose and galactose have also been shown to be weak effectors of the

Crabtree effect. In the metabolism of galactose by *S. cerevisiae*, it is apparent that the preferred route is respiratory (De Deken 1966).

Other metabolic effects have also been described in yeasts including the Pasteur effect, Custers effect and Kluyver effect. The Pasteur effect has been described as the increased rate of utilisation of glucose under anaerobic conditions compared with aerobic conditions and many attempts have been made to study the regulation of the glycolytic pathway that may allow for this effect. However, this effect only occurs in *S. cerevisiae* under conditions of nutrient limitation, in resting cells or when the glucose concentration is low (below 5mM) (Lagunas 1979). Attempts to identify the controlling step of the glycolytic pathway that could be responsible for this alteration of glycolytic rate has lead to the identification of fructose-2,6-bisphosphate in regulating the balance between gluconeogenesis and glycolysis. This metabolite activates the glycolytic enzyme, phosphofructokinase, and deactivates the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hers *et al* 1982 cited in Zimmerman and Entian 1998). It has now been shown that the fate of glucose in *S. cerevisiae* is extremely tightly regulated. Attempts to increase glycolytic flux by overexpressing phosphofructokinase have shown that glycolysis is not controlled by a single step (Davies and Brindle 1992). Similarly, by overexpressing pyruvate decarboxylase increased fermentation need not necessarily occur (Van Hoek *et al.* 1998a). However, in studying the effect of growth rate on glycolytic enzymes, only phosphofructokinase and pyruvate decarboxylase showed increased activity with increasing growth rate, leading the authors to propose overexpression of both enzymes to increase fermentative activity of yeast (Van Hoek *et al.* 1998b). It is pertinent to note that producing yeast by aerobic processes, (fed-batch

culture with molasses as substrate, as described above) may be detrimentally affected by improving a yeasts fermentative performance in such a fundamental manner.

The other effects on yeast metabolism mentioned discussed above are not, in general, applicable to *S. cerevisiae* strains. The Kluyver effect is defined as the inability of some yeasts to use disaccharides anaerobically, despite utilising the component monosaccharides anaerobically. A recent study of this effect has shown (Weusthuis *et al.* 1994b) that in a Kluyver positive yeast (*Candida utilis*), oxygen appears to pay a key role in regulation of the enzyme pyruvate decarboxylase. The authors of this report also give a much clearer definition of the Kluyver effect as "the inability to ferment certain disaccharides to ethanol and carbon dioxide even though respiratory metabolism of the disaccharides and alcoholic fermentation of the component hexose(s) can occur" (Weusthuis *et al.* 1994b).

The other effect referred to is the Custers effect which is specific to *Dekkera* (*Brettanomyces*) species, which are important in the fermentation of the Lambic beers (Belgium). This effect sees the yeast ferment faster under aerobic conditions than anaerobic conditions (Zimmerman and Entian 1998; Walker 1998).

1.7. Aims and Objectives

The *S. cerevisiae* genome has now been completely mapped and many studies have been undertaken to genetically engineer yeasts with attractive industrial characteristics, (Klein 1998); (Van Hoek *et al.* 1998a). However, such studies are unable to identify the effects of genetic manipulations on yeast physiology. Furthermore,

effects on industrial yeast strains are unknown and so better understanding of the parent strains' behaviour would aid in selecting the correct genes to manipulate. In short, the information on the cellular physiology of industrial yeast strains is very limited.

Research on yeast physiology in industrial applications has generally concentrated on fermentative applications, as has most of the genetic enhancement work. This is due to the huge economic significance of the fermentation products of *S. cerevisiae*, e.g. beer, bread, wine, spirits and bioethanol. However, as discussed in section 1.2, the increasing use of *S. cerevisiae* as a food additive, means that greater understanding of its aerobic growth characteristics is required, especially with regard to the industrial propagation of yeast biomass for yeast extracts.

The general aims of this project were to investigate the physiological characteristics of a baker's yeast strain of *S. cerevisiae* in aerobic continuous culture. Specific aims were to establish the effects of glucose-limitation on growth and metabolism of the yeast and to evaluate effect of nutrient perturbations on the yeast steady state, particularly with regard to organic and inorganic intracellular macromolecular components of relevance. It was hoped from the outset that the research would provide fundamental cell physiological knowledge on yeasts specifically grown for applications in the food industry.

CHAPTER 2 - MATERIALS AND METHODS

2.1. Yeast culture maintenance

The yeast used predominantly in this study was *Saccharomyces cerevisiae* GB 4918, a diploid strain of baker's yeast. This was supplied as a freeze dried culture by Quest International, Menstrie. This master culture was revived in 10ml malt extract broth and incubated at 30°C for 2-3 days. Dilutions of this culture were then plated out on malt extract agar plates and incubated at 30°C for 48h. Resultant single colonies were aseptically removed and streaked onto malt extract agar slopes, incubated for 48h at 30°C, then stored at 4°C until required for later experiments. Another 13 yeasts are described in this thesis. These are descibed in the appropriate Chapters (Chapter 4 and 5), where the full relevance of the yeasts is explained.

2.2. Batch propagations

In order to ascertain the precise level of glucose that was required to maintain glucose-limited chemostat cultures of *S. cerevisiae*, a series of batch propagations were initially performed as follows.

2.2.1. Growth media design and preparation

The defined medium that was employed was a hybrid version of both Edinburgh Minimal Media (EMM3 - Mitchison 1970) and a defined yeast propagation medium supplied by Quest International, Menstrie. The resulting medium, termed QEMM3, comprised of inorganic salts, vitamins and trace elements which were all supplied at levels well above those found in EMM3 (Table 2.1.).

Table 2.1. Yeast propagation defined medium (QEMM3).

Carbon source	Glucose	variable
Nitrogen and sulphur source	Ammonium sulphate	5g/l
Phosphate source	Ammonium dihydrogen phosphate	2.84g/l
Potassium source	Potassium chloride	2g/l
Magnesium source	Magnesium sulphate heptahydrate	1g/l
Calcium source	Calcium chloride dihydrate	30mg/l
Zinc source	Zinc sulphate dihydrate	0.8mg/l
Trace elements	Potassium iodide	0.15mg/l
	Manganese sulphate	0.6mg/ml
	Copper sulphate	60µg/ml
	Citric acid	1.5mg/l
	Molybdic acid	0.24mg/l
	Ferric chloride	0.3mg/l
	Boric acid	0.75mg/l
Vitamins	Nicotinic acid	40mg/l
	Inositol	40mg/l
	Calcium pantothenate	4mg/l
	Thiamine -HCl	1.6mg/l
	Pyridoxine -HCl	1.6mg/l
	Biotin	40µg/l

The inorganic components of this medium were prepared individually and the trace elements and vitamins were produced as 1000-fold and 250-fold filter-sterilised stock solutions, respectively. This meant that whether they were being utilised for batch or continuous propagations the levels of nutrients required could be consistently added to every medium formulation. These stock solutions were stored in sterile containers and maintained at a temperature of -20°C. A range of glucose concentrations were chosen (0.1g/l - 10g/l) which were known to be within the required growth limits of glucose by baker's yeast. These glucose concentrations were added to 90.9ml of deionised water (16.7 megohms-cm, Barnstead Nanopure II water purification system) in 250ml baffled

Erlenmeyer flasks, which were then autoclaved at 121°C at 15psi for 15 minutes. This allowed the aseptic addition of the sterile components mentioned earlier to a final volume of 100ml. The reason for autoclaving the glucose and the inorganic fractions separately was to avoid the condensation reactions that can take place between glucose and amino-groups, known as the Maillard reaction, which results in a browning of the medium. The trace elements and vitamins were also aseptically added at this time.

2.2.2. Inoculum Preparation

In glucose-limitation experiments it was necessary to try and achieve relatively similar initial cell densities in experimental cultures. This allowed the growth in each of the flasks to be compared accurately and determined if glucose was a limiting factor on yeast cell growth.

This was achieved by inoculating 100ml of QEMM3 in baffled Erlenmeyer flasks with a loopful of cells from a stock slope of baker's yeast. QEMM3 was used with a glucose level of 5g/l. This facilitated rapid growth in the seed culture. This culture was then grown overnight with agitation at room temperature, or for a maximum of 24h. Cell numbers were then determined in order to equalise yeast inocula to be used in seeding glucose-limited cultures. Equal aliquots were then centrifuged at 1,341g for 10min in sterile centrifuge tubes, and the pellet washed once by resuspension in sterile dH_2O. These washed pellets were then resuspended in 1ml aliquots of the sterile medium prior to inoculation into experimental culture flasks.

2.2.3. Analysis of glucose-limited growth

2.2.3.1. Cell Numbers

In initial glucose-limited experiments the main parameter of interest was cell numbers as an interpretation of growth rate of the cells. This was done using a Coulter counter (Coulter counter multisizer II, Coulter Electronics, Luton, Bedfordshire). Electronic measurement of the cell numbers enabled quick cell number determination at frequent (hourly) time intervals. To facilitate accurate cell number determination it was first necessary to sonicate the samples for 1 min in a sonicating water bath (Camlab), to disperse any clumped yeast cells. The sample was then appropriately diluted to provide a counting sample that contained less than 40,000 cells/ml. This number represented approximately where the error margin used by the counter to adjust the results was acceptable at 5% or below. Any counts which resulted in an error above this threshold resulted in a further dilution.

2.2.3.2. Ethanol determination

Ethanol determination was used as a secondary method to show which glucose concentration was limiting for respiratory growth. The production of ethanol along with significant rises in cell numbers would signify non-limiting concentrations of glucose. Rising cell numbers without the concomitant appearance and rise in ethanol would be defined as glucose-limited, as opposed to cultures in which growth was slowed by the deficiency of glucose. Ethanol was determined using a Hewlett-Packard Gas Chromatograph 5710A. The separation was carried out in a 2 metre steel column packed with poropak Q (mesh 80-100), at a temperature of 120°C, using nitrogen gas as the carrier gas. Detection and quantification was carried out by a flame ionising detector

(FID) set at 200°C and linked to a Hewlett-Packard integrator 3396A. The calibration was of an internal standard type with the quantification being based on peak area. The integrator was calibrated using a 1% ethanol standard with 5% isopropanol as internal standard. A one-point calibration was all that was necessary to calibrate the GC as the characteristics of the column and standard resulted in linear relationship, beyond 1% v/v and up to 5% v/v ethanol concentration.

2.3. Chemostat cultures

2.3.1. Chemostat operation

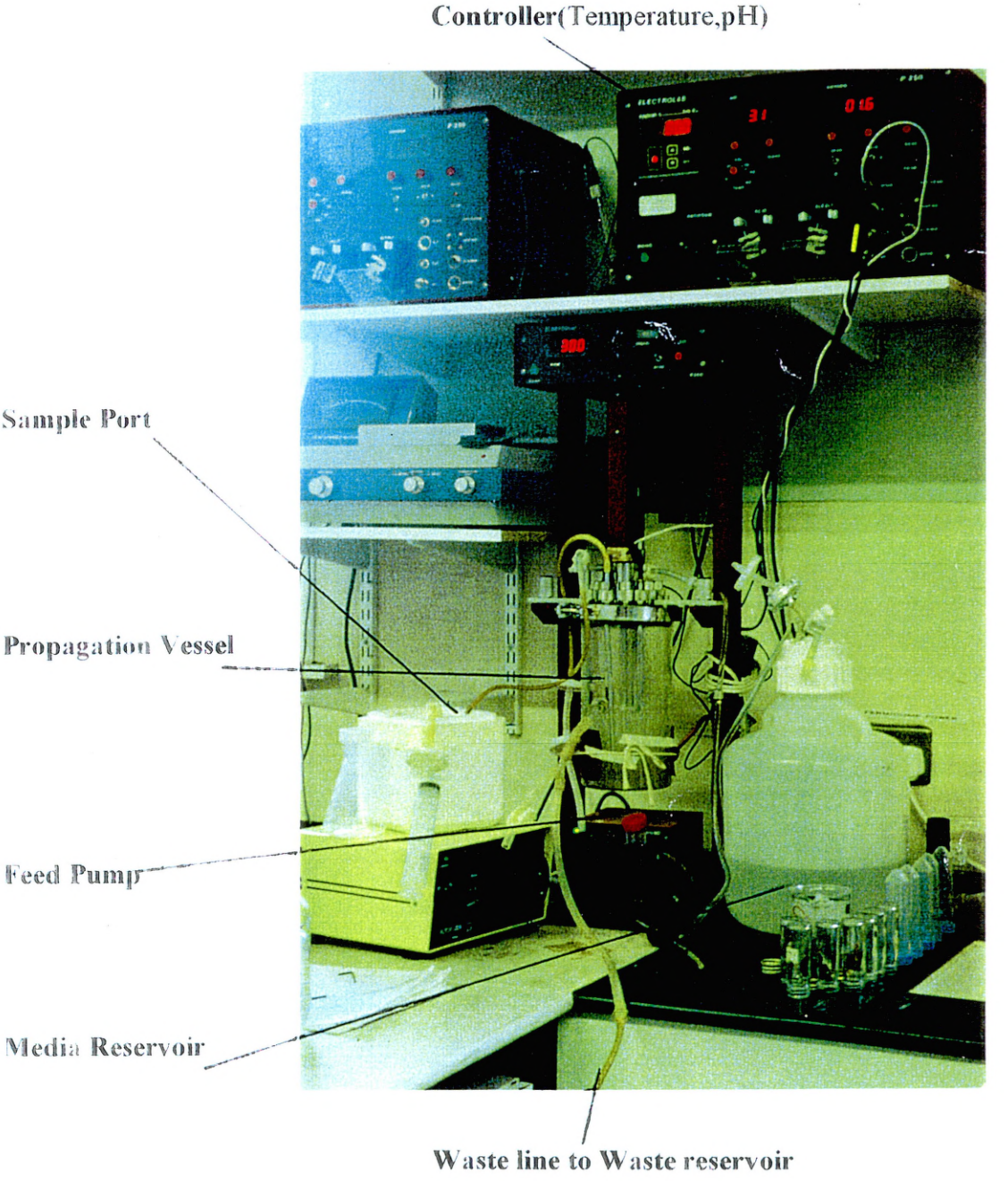
2.3.1.1. The Chemostat

The design of the chemostat utilised in this research evolved over a period of time. Originally the design of the system was such that it would allow more on-line analysis to be carried-out and to allow the use of other methods to determine the steady state conditions prevailing in the chemostat. These included the continual monitoring of culture density using a flow-through cuvette in a spectrophotometer linked to a pen recorder; cell density was calibrated using a dry weight versus culture absorbance graph. This was attempted but a variety of technical restrictions resulted in it's use being abandoned.

The final chemostat design that was used is shown in Figure 2.1. The various components include: the vessel itself (a 2l glass fermenter, Electrolab, Tewkesbury, UK, working volume = 1155ml), a 10L carboy (Nalgene, UK) serving as the sterile feed medium reservoir connected using food standard sterile tubing broken with two, one-

way check-valves (Nalgene, UK). The feed medium was pumped using a Watson Marlow 101U pump, the overflow (weir-overflow adaptation) passed through tubing into a sterile 20L carboy (Nalgene, UK), a sample device (adaptation of the original construct provided by Electrolab, UK) allowed better and safer sample extraction from the vessel without any major risk to the sterility of the system was utilised. Air was supplied by an air pump (KNF-Neuberger, 5L/min) linked to the vessel sparger by sterile Neoprene tubing (Mackay and Lynn, UK), which was split into two sections to allow effective filtration of the air supply. The first section (nearest the pump) was split into two and passed through two 0.2μm Whatman air filters (Whatman, UK), which allowed the two primary filters to be changed at any time during chemostat operation without causing any disruption to the air flow into the vessel and therefore to prevent disturbing the steady state of the cells. The second section leading to the sparging finger itself was reunited before passing through another two 0.2μm air filters (Whatman, UK). These were left in place until a set of experiments were carried out, when it was deemed acceptable to perturb the culture as a settling period was required before the chemostat reached steady state conditions. The result of this splitting of the air line and the filtration devices present within it was a drop in air flow rate from the 5 l/min measured at the pump outlet to 3.5 l/min at the sparger.

Figure 2.1. The chemostat apparatus.



2.3.1.2. pH monitoring

Culture pH was measured using a Broadley-James Fermprobe connected to an Electrolab P-350 control and monitoring unit (Electrolab, UK). Calibration of the pH probe was carried out at a temperature of 25°C and by immersing the probe in a neutral buffer (pH 7) and then in an acidic buffer (pH 4). Calibration of the probe in an alkaline buffer was considered unnecessary as the chemostat would not be operating under alkaline conditions.

2.3.1.3. Dissolved oxygen monitoring

The levels of dissolved oxygen in the chemostat were monitored using a polarographic Uniprobe oxygen electrode (Electrolab, UK). The oxygen electrode was calibrated at 0.00% O_2 by sparging N_2 through the air line under normal operating conditions for 1h. The air line was then attached to the air pump and the chemostat then subjected to aeration under normal conditions for 1h before the range control was used to bring the control readout to 100.00%. All appropriate O_2 recordings were then expressed relative to the 100% level produced under the calibration conditions.

2.3.2. Media preparation - Chemostat

QEMM3 (see section 2.2.1) was used in all chemostat studies. Media were, however, prepared differently due to the quantities of inorganic nutrients required and the fact that the vitamin mixture could not be sterilised by autoclaving. In the culture vessel the inorganic compounds were added to 980ml of deionised water, along with the pre-prepared, sterile-filtered trace elements and autoclaved at 121°C for 20min with all the vessel components in place (e.g. pH probe, O_2 probe, feed line, overflow lines,

sample lines etc. (Figure 2.1.)). The amount of glucose required was dissolved in 16ml of deionised water and sterilised separately in a universal bottle that was complementary to the sampling device of the vessel. Once this was cooled 4ml of sterile-filtered vitamin mix was added to it and then this was injected into the sterile, cool culture vessel through the sample device (Figure 2.1).

2.3.3. Media Preparation - Feed reservoir

The medium in the feed reservoir was prepared in a similar way to that in the vessel except that it was produced in larger quantities and sterilised in a longer autoclave programme, 121°C for 1h. The preparation of feed medium also utilised a pressure-based sterile filtering system (Sartorius, UK), with a 0.2μM pore size cellulose nitrate filter as an alternative media sterilising method. The use of the pressure filtration system had many advantages over the autoclaving procedure, including the speed at which the media was sterilised and the minimisation of possible routes of infection. Disadvantages of the system were limited mainly to the fact that filter failure was only identified after the media had been pumped into the feed medium reservoir.

2.3.4. Chemostat inoculum

In chemostat experiments seed flasks were used in a similar way to that described earlier in this Chapter, except that the calculated volume of seed culture was added directly to the chemostat without the washing step. This was deemed unnecessary as the culture medium from the seed flask would be diluted by the chemostat medium and the fact that the effect of dilution rate on the culture would remove any residual effect of the seed medium by the time the chemostat reached the steady-state.

In later chemostat experiments it became necessary to add an extra precautionary step in the seed stage. This involved streak plating a loopful of cells from the stock slope of baker's yeast on a malt extract agar plate. This was left to grow at room temperature for 48h when single colonies became apparent. A single, representative, colony was then picked from the agar and inoculated into the medium in the seed flask, which was then allowed to grow for 24h before being used to inoculate the chemostat.

2.3.5. Chemostat sampling procedure

The fermenter sampling device was adapted, using a suitable length of tubing, to act as both a sampling device and as an inoculation device. This device consisted of a piece of stainless steel tubing (located in a 12mm vessel port) to which was attached a length of tubing that connected to the sampling head device. The sampling head device, as shown in Figure 2.1, consisted of a stainless steel cap with a side-arm and a stainless steel tube passing through it. The stainless-steel tube was connected to the tubing that lead to the sample tube in the fermenter at one end and another, smaller length of tubing was attached to the other end. This small piece of tubing was the adaptation that allowed the use of the sampling device as an inoculation port. To this bottom end of the cap a sterile universal bottle could be screwed in to allow either collection of culture sample or aseptic transfer of yeast inoculum, glucose/vitamin mixes etc. A sterile air filter (0.2μm Whatman hepavent) was attached to the side-arm which facilitated aseptic transfer (using a 50ml syringe). Medicine flat bottles, capable of holding larger sample volumes, were also attached to the sample cap, when appropriate.

2.4. Analytical methods

2.4.1. Cell Numbers and Budding Index

Cell numbers in the chemostat were measured (at least once every 24h) using a Neubauer improved haemocytometer. The counts were performed in a modified version of the cell counting method accepted by the Institute of Brewing (IOB, 1991). Cells were counted from 10 of the large squares in the haemocytometer, in a pattern consistently applied to each sample. All cells were counted and buds only included as separate cells if they were considered to be about two-thirdsof the size of the mother. This was carried out for two samples and the number of cells per millilitre of culture calculated using the calculation: total cells counted/10x250x1000xdilution factor. This calculation accounts for the volume of the haemocytometer ($0.004mm^3$), which then has to be multiplied by 1000 as there are $1000mm^3$ in 1ml. The average number of cells counted for each sample was in the range 200-300cells/ml (for statistical accuracy). Budding index was carried out as a percentage of the total population by dividing total number of buds counted by the total number of cells counted (x100).

2.4.2. Cell Sizes

Cell sizing was carried out using a Coulter counter multisizer II (Coulter Electronics, Luton, UK.) using a 200µl sample dispersed in 20ml of Isoton II solution (Coulter Ltd). Samples were passed through a 100µm aperture probe until a statistically appropriate number of cells (250,000) had accumulated. The Coulter Counter software (Accucomp, Coulter UK) was then used to analyse the cells and provide the mean cell volume of the culture.

2.4.3. Dry weight of propagated yeast

To obtain a satisfactory dry weight of yeast cultures a 5ml sample was vacuum filtered onto pre-weighed Whatman GF/C filter papers (pore size 1.2μm, diameter 47mm). Samples were then washed three times with deionised water and the filter was transferred to a Mettler Infra-Red Moisture Analyser (Mettler Toledo, UK). Although the infra-red dryer was capable of providing the dry weight of the sample, all filter papers and dried samples were transferred to a desiccator prior to weighing on a 4-decimal place balance. This was deemed necessary due to the small size of the pellets obtained from 5ml samples. This was carried out in duplicate and all intracellular analyses (RNA, trehalose, protein, glycogen) were expressed in relation to yeast dry weight.

2.4.4. Trehalose and Glycogen Analysis

As discussed in Chapter 1, these two carbohydrates appear to have differing roles in yeast cell physiology. Several methods to extract and analyse trehalose and glycogen in yeast cells have been published. Before starting the chemostat experiments, it was deemed prudent to attempt several methods to ascertain which would be most suitable for use in this research. These methods are now described and the results of a trial run of each is also presented in order to justify the final selection.

Trehalose assay 1 (Trevelyan and Harrison, 1956)

A frequently-cited method of yeast trehalose analysis is the one originally described by Trevelyan and Harrison (1956). This method was developed while the authors worked at Distillers company Ltd (DCL) Glenochil yeast factory at Menstrie in 1956, which is now run by Quest International. This method has been widely reported in

recent literature and has been used by some of the major groups researching trehalose and it's role in yeast physiology (e.g. Mansure *et al.* 1997). The method (Trevelyan and Harrison 1956) involves incubation of yeast cells in 4ml of 0.5M TCA (trichloroacetic acid) in an ice bath for 20min. The cells are then centrifuged for 10min at 4000rpm and the supernatant removed and stored in a clean tube at 4°c. The incubation in TCA and centrifugation is repeated twice with supernatants being pooled prior to cold storage. The pooled supernatants are then subjected to the anthrone method for the determination of sugars (Munro and Fleck 1966). Due to concerns regarding safety of the anthrone method and the practicality of other equipment at the disposal of the research it was deemed sensible to alter the determination method to utilise HPLC equipment. This equipment was already being used for sugar analysis and was capable of determining trehalose.

Trehalose assay 2 (Quest International)

The second method that was tested was a method provided by the analysis laboratory of Quest International, Menstrie. This method was designed for use on yeast cakes or slurries, and doubts were raised to whether or not it would be suitable for the pellet sizes that were obtained from preliminary chemostat experiments. The method involves resuspending 1g of wet weight of yeast in a graduated plastic centrifuge tube and then made up to 12ml with deionised water. The resulting slurry was then placed in a constantly boiling water bath for 10min. After cooling and centrifugation (1,341g for 10min), the supernatant was removed for analysis by HPLC. By coincidence, the HPLC column used by Quest International (HPX-87H, Bio-Rad, UK) was the same as that used in this research and a valuable method of verifying results was available.

Trehalose assay 3 (Yoshikawa *et al.* 1994)

This method involved refluxing a yeast sample in an alcohol-water mixture for 20min and then analysing the resulting supernatant for trehalose (Yoshikawa *et al.* 1994). This method was investigated due to its relative simplicity and the suitability of the procedure for the laboratory in which the work was carried out. In preliminary work, however, it was found that this method was too cumbersome as it required many separate reflux apparatus and was far too time consuming.

The results from a preliminary trial involving all three methods and a slight variation of Quest International's boil method are shown in Table 2.2. The yeast used in this trial was a commercial spray dried yeast (*S. cerevisiae*) with a trehalose content stated to be about 4% dry weight (although the content was expected to be lower due to the age of the yeast). Each method was applied to a similar weight of dried yeast in quadruplicate and the mean of all four samples are presented here. The cake and boiled method is a variation on the boil method where the cells are centrifuged and resuspended in water before being boiled. In the other methods the cells were also treated in this manner, except for the boil method where once the dried yeast was reconstituted the cells were immediately placed in a boiling water bath.

Table 2.2. Preliminary results of four trehalose extraction methods.

Sample	Boil	Cake + Boil	TCA	Ethanol reflux
Dry wt(mg/ml)	173	154.8	178.2	146.2
Trehalose(%)	2.651	2.25	0.227	1.3215

As is apparent from these results the boiling methods appeared to be the most effective methods for extracting trehalose from the cells. The ethanol reflux method also

appeared to be a better method than the TCA extract method but was wasteful of both time and resources. As a double check, the TCA and boiled extracts were taken to the analytical laboratory at Quest International where a similar HPLC system was in use and the same discrepancy was noticed between the TCA and boil methods. This led to a final method of ensuring a reliable, reproducible method of trehalose analysis was carried out by gathering 8 samples of baker's yeast, from the same yeast propagation at Quest International's Glenochil yeast factory. Each sample was measured to give a wet weight of yeast of 10g and half of this was then placed in graduated, plastic centrifuge tubes. The wet yeast cake was then made up to 10ml volume with dH_2O. The remaining half of the yeast sample was taken for dry weight analysis. The sample in the centrifuge tubes were then placed in a boiling water bath for 10min. After this the tubes were cooled before being centrifuged at 1,341g for 5min and the supernatant collected. The supernatant was separated into two aliquots and frozen. Half was transported back to Dundee to be analysed on the equipment described in this thesis, the other half remained in Menstrie to be analysed by the similar HPLC system mentioned earlier.

As can be seen from the results in Tables 2.3 and 2.4 there was no real difference between the two analytical systems involved, the error between them is only slightly higher than 4%. The largest deviation occurred in the samples analysed by the HPLC system used in this thesis, but even then the standard deviation was 4.5% as compared to 1.3% measured in the HPLC equipment in the Menstrie. The major reason for the difference in the results obtained is most likely due to the age of the columns being used, the column used in Dundee being older than the column in use at Menstrie.

Table 2.3. Results of Dundee trial analysis of yeast trehalose by the boil method

Dundee sample	HPLC mg T/ml	Correct for acid	dilution factor	trehalose total mg	wet wt g	dry wt g	Trehalose gT/100g YDW
1	1.315	1.46	10	146.11	4.99	1.54	9.49
2	1.200	1.33	10	133.33	5.08	1.56	8.52
3	1.300	1.44	10	144.44	5.00	1.54	9.38
4	1.210	1.34	10	134.44	4.98	1.53	8.77
avg.							9.04

T = trehalose; YDW = yeast dry weight

Table 2.4. Results of Menstrie trial analysis of yeast trehalose by the boil method

Quest sample	HPLC mgT/ml	correct for acid	dilution factor	trehalose total mg	wet wt g	dry wt g	Trehalose gT/100 YDW
1	13.25	14.72	1	147.22	5.05	1.55	9.47
2	13.18	14.64	1	146.44	5.08	1.56	9.36
3	13.28	14.76	1	147.55	4.99	1.54	9.59
4	12.84	14.27	1	142.67	4.99	1.54	9.27
avg.							9.42

T = trehalose; YDW = yeast dry weight

The results from these trials made the final choice of extraction method for both trehalose and glycogen content of *S. cerevisiae* a rather obvious one. The appearance of glycogen in the extracts was valuable as this meant that the boil method could also be applied to this parameter. Thus, chemostat samples, (10ml) were centrifuged and residual pellets resuspended in 1ml of dH_2O and placed in a constantly boiling water bath for 10 minutes, before being cooled and centrifuged and the resulting supernatant was collected for analysis by HPLC. The HPLC system used included a Bio-Rad pump (model 1330, Bio-Rad, UK) and column heater and utilised a Knauer Differential Refractometer for identification of the analysed compounds. The separations were carried out using a Bio-Rad Aminex HPX-87H column with 0.005M H_2SO_4 as a mobile

phase. The results were interpreted by a Hewlett-Packard integrator (3396A) which had been calibrated using a three-point calibration. Standards containing glycogen, trehalose and glucose were analysed at concentrations of 0.5, 1.0 and 2.0mg/ml.

2.4.5. Protein Analysis

Protein content of yeast cells was assayed by microcentrifuging a millilitre of cell suspension (3 minutes, 10,000rpm), washing with dH_20 before resuspending the pellet in 1ml of 1M NaOH and incubating at 60°C for 1 hour. The result was a clear hydrolysate which contained a completely solubilised cell preparation. Aliquots (100µl) were neutralised with 100µl of 1M HCl. Aliquots of neutralised hydrolysates (100µl) were placed in a sterile Eppendorf tube to which 1ml of Coomassie Blue reagent (Pierce ltd, UK) was added, mixed thoroughly and then left for 2-3 minutes. The absorption of the sample was then read against a water blank at 595nm in a LKB spectrophotometer and the optical density (OD) of the sample read off against a freshly prepared calibration curve using bovine serum albumin (BSA) as standard (provided with the Coomassie Reagent Kit, Pierce).

2.4.6. Free Amino Nitrogen (FAN) Analysis

Free amino nitrogen measurements were carried out to ascertain how cells utilised the nutrient resources available to them and was a measure of yeast protein metabolism. After reviewing the literature it was decided to employ a method described for FAN analysis of beer and wort by Lie *et al* (1973) (IOB, 1991). Cell extracts produced in the protein determination method described above (section 2.4.5) were mixed with a ninhydrin based reagent (see Table 2.5.) and optical densities measured at

570nm in an LKB spectrophotometer to determine levels of free amino nitrogen.

Table 2.5. Reagents for yeast FAN analysis

a) Colour reagent.			b) Diluting reagent.	
disodium phosphate	10g/100ml		water(dH_2O)	600ml/l
dipotassium phosphate	6g/100ml		ethanol(96.5%)	400ml/l
ninhydrin	0.6g/100ml		potassium iodate	2g/l
fructose	0.3g/100ml			

Diluted alkali hydrolysates (2ml) (see section 2.4.5) were added to 3ml of the colour reagent, stoppered with a glass marble and placed in a constantly boiling water bath for precisely 16 min. The tubes were then placed in a water bath at 20°C for 20min, prior to addition of diluting reagent (5ml see Table 2.5b) and absorbance measurements of the mixture read at 570nm within 30min. The assay was standardised by treating a 1:100 dilution of a glycine solution (102.7mg/100ml) in the same way as experimental samples. The absorption reading that was recorded was equivalent to 2mg/l of α-amino nitrogen.

2.4.7. Ribonucleic acid (RNA) analysis

There are many ways of determining the levels of RNA in cells, most of which are now related to extracting good quality RNA for molecular biological techniques such as Northern blotting. However, for the purpose of the present research it was decided that quality of RNA was not a priority and that a more 'brutal' method could be employed to extract and determine levels of total cellular RNA present in *S. cerevisiae*.

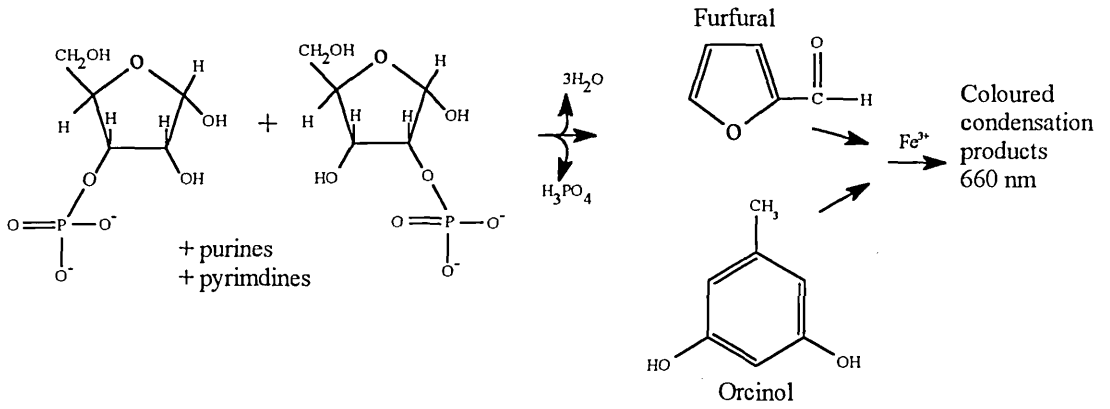
A search of the literature revealed that the most appropriate method to be one developed by Schmidt and Thannhauser (1945) (Munro and Fleck 1966) and adapted by Quest International, before being further modified for this research. This method involved washing the cells in various concentrations of $HClO_4$, in an ice-bath, and pooling the supernatants. Final volumes, in the adapted method, were too small to be measured using absorbance at 260nm and so were quantified using the orcinol method for furan-based sugar determination (Munro and Fleck 1966).

The pellets collected from the chemostat culture samples were washed with 0.25ml of ice cold 0.6N perchloric acid and allowed to stand for 10min before being centrifuged (1,341g, 5min), washed twice with 0.4ml of ice cold 0.2N perchloric acid and dried carefully be inverting Eppendorf tube onto a piece of filter paper. Once this had removed excess acid, 0.4ml of 0.3N potassium hydroxide was added to the pellet and the tubes were then placed in a 37°C incubator for 1hr. The digest was then allowed to cool in ice before 0.25ml of perchloric acid was added to precipitate out protein and DNA. This was left for 10min on ice before being centrifuged at 1,341g for 5min. The supernatant (0.65ml) from this stage was retained. The resulting pellet was then washed twice with 0.175ml of 0.2ml of perchloric acid, the washings retained and added to the volume obtained after the precipitation stage.

After the extraction procedure a final volume of 1ml was obtained and this was analysed for furan content using the orcinol method developed by Bial (Munro and Fleck 1966). This method involved dissolving 0.3g of ferric chloride in concentrated hydrochloric acid. To this solution 3.5ml of orcinol reagent was added. The orcinol

reagent was (fresh for each analysis) made by dissolving 6g of orcinol in 100ml of a 96% ethanol solution. This colour reagent was kept in a light-proof container as the orcinol reagent was quickly degraded by light. The samples were assayed in duplicate with 0.5ml aliquots being made up to 2ml before the addition of 3ml of the colour reagent. The samples were then placed in a boiling water bath for 20min along with a range of RNA standards made up in a similar $KOH/HClO_4$ solution as the samples, with 100mg of RNA (type VI from Torula yeast Sigma, UK) dissolved in 100ml of dH_20. This solution was then diluted to provide a range of standards from 1µg/ml to 1000µg/ml. After the samples and standards had been allowed to cool the absorbance of all tubes was measured at 660nm in a LKB spectrophotometer. A calibration curve was plotted using the results obtained for the standards and the water $KOH/HClO_4$ blanks. The reaction involved in this colourimetric procedure is shown in Figure 2.2. (Clark and Switzer 1977).

Fig 2.2. Reaction of orcinol with ribose-subunits of RNA in Bial's test for furan.



2.4.8. Verification of glucose-limited steady state growth of *S. cerevisiae* in chemostat culture

During the operation of the chemostat it became necessary to show that glucose was in fact limiting and that no ethanol was being produced by the yeast. The assumption being that if the cells were not glucose-limited then ethanol would also be produced as a result of the respiratory bottleneck at pyruvate (Fiechter and Seghezzi 1992). It also became apparent that keeping supernatants removed from cellular samples was of value in studying the effect of experimental conditions on the extracellular environment of the yeast cells. This could be through the appearance of a compound in the supernatant that could not be attributed to whatever experimental change was attempted, e.g. the presence of malic acid when malic acid had not been presented to the cells. These supernatants were derived from 1ml samples obtained for protein, RNA or FAN analysis, and subjected to HPLC analysis. It was established during this procedure that various inorganic constituents (e.g. ammonium sulphate, ammonium dihydrogen orthophosphate, and potassium chloride) of the QEMM3 interfered with the analysis. The main peaks interfered with were glycogen and tartaric acid. This analysis had the double benefit of determining glucose as well as ethanol in the same sample. Although this method may not be as sensitive as some of the previously described methods (Brown *et al.* 1987) it constantly provided residual glucose levels of >35μg/ml accounting for less than 3.5 % of the available glucose. Results obtained from this analysis along with those obtained from yeast dry weight analysis allowed a yield to be calculated which determined the efficiency of the chemostat biomass production yield. Theory dictates (Weusthuis *et al.* 1994a) that only 50% of available sugar can be utilised by *S. cerevisiae* to produce biomass as the rest will be lost as CO_2 and H_20. Therefore, growth on 1g/l glucose yields a maximum

theoretical value for biomass production of 0.5g/l. This figure is impossible to achieve due to the maintenance energy requirements of the yeast cells which are required for the basic upkeep of the cells without contributing to any reproductive function.

2.4.9. Statistical Analysis

All of the experiments in this project were designed to allow statistical analysisand were performed in, at least, duplicate (Heath 1995). All analyses of samples collected from these experiments was carried out in triplicate, and the data presented in this thesis represents the mean of these data sets.

CHAPTER 3 - PHYSIOLOGY OF GLUCOSE-LIMITED BAKER'S YEAST CELLS CONTINUOUSLY CULTURED IN A CHEMOSTAT

3.1. Introduction

One of the first aims of this research was the establishment of a defined medium that would be appropriate for the growth of baker's yeast under glucose-limitation. In *S. cerevisiae* this is a relatively simple process because under aerobic conditions, *S. cerevisiae* will respire when the glucose levels are sufficiently low. Reports have suggested that this level varies from strain to strain. In studying expression of the glucose-repressed enzyme invertase, Toda *et al* (1982) has reported a threshold figure of around 0.14mM in phosphate-limited continuous cultures of *S. cerevisiae*, while 0.12mM was believed to be sufficient to exhibit repression. Once this level is surpassed then the yeast will begin to utilise the glucose in a respiro-fermentative fashion instead of solely by respiration. This would be manifest by a rapid increase in growth rate and the production of ethanol (see Chapter 1).

Batch culture experiments were set up using a series of baffled Erlenmeyer flasks in which differing amounts of glucose were used. It was hoped that results obtained from growth determinations would lead to a level of glucose at which the cells grew but at which they did not ferment (i.e. produce ethanol). This would then allow for a set of experiments to be designed that would allow a chemostat culture of the baker's yeast to be studied for a variety of intracellular macromolecules: protein, RNA, trehalose and glycogen. The limitation of glucose in the growth medium allowed the investigation of other parameters, including responses to other nutrient sources, which will be expanded

upon in Chapters 5 and 6.

3.2. Glucose-limited yeast physiology in batch culture

3.2.1. Experimental approach

As discussed in Chapter 1, the majority of industrial media are complex and often technically difficult to handle. It was therefore, apparent that chemically-defined media would be more appropriate for experiments involving fundamental studies of the effect of nutritional factors on the physiochemical make-up of yeast cells.

The hybrid medium that was constructed for these experiment is described in full in Table 2.1 (chapter 2). The medium is designed so that only glucose should be limiting. The only other factor that was expected to become limiting during batch propagation trials was that of oxygen transfer, which would be indicated by the accumulation of ethanol. This effect can also be brought about by other limiting nutrient conditions, most noticeably, vitamin or phosphate deficiencies.

The agitation rate (125rpm and 150rpm) used in these batch propagations almost certainly effected the rate of oxygen transfer in shake flasks, although the use of baffled flasks should partially circumvent this. The design of the experiment was to determine which level of glucose was limiting. Such an identification at this rate of agitation would mean that a lower glucose concentration may be fermented in the baffled flask environment, due to poor oxygen transfer. However, oxygenation conditions in the chemostat were superior to batch culture as air would be directly supplied, removing this limitation.

3.2.2. Results and Discussion

Glucose-limitation was employed as the basis of all the continuous cultures since this would enable control of biomass and energy metabolism. The methods used in these studies were as described in Chapter 2. The cell counts (by Coulter counter) were used to calculate yeast growth rate under the different glucose concentrations.

Figures 3.1 and 3.2 show that the growth of *S. cerevisiae* was not limited at glucose levels above 2g/l. Cell numbers were observed to have more than doubled in 8 hours. Levels of ethanol at this glucose concentration (2g/l) were too small to be reliably quantified at that time but levels were detected at the same sample point as at the onset of logarithmic cell growth. This was not the same for the other glucose concentrations studied in Figure 3.1. Omitting the micronutrient fraction from the medium seemed to slightly hinder the growth of the yeast, especially under glucose limiting conditions (see Figures 3.1 and 3.2). However, the effect of omitting the micronutrient fraction may have a greater effect on a culture that is growing over a significantly longer period of time, such as in a chemostat, and this was the reason for the decision to include the micronutrient fraction in the medium for continuous culture. The micronutrient fraction includes inorganic factors such as manganese and iron which have important roles in yeast function as discussed in Chapter 1.

Any change in cell numbers observed when cultures were clearly glucose-limited can be attributed to variations between sample and experimental error. Cells grown in 1g/l glucose with added micronutrients exhibited slightly slower growth, compared with the 1g/l culture which lacked the micronutrient fraction. Both these cultures did not

exhibit any ethanol formation, suggesting that both cultures were respiratory. As can be seen from the comparison of growth rates in Figure 3.2, there was a greater reduction in growth between 2g/l and 1g/l than between any of the other glucose concentrations. This loss of growth rate also appeared to be accompanied by a lack of fermentation in the slower growing cells. The data presented in Figure 3.1 were used to calculate growth rates and mean generation times using the following equations:

log cell number at time t_1- log cell number at time t_0 / time t_1 - time t_0 = $\mu(h^{-1})$,.

specific growth rate or simply $dx/dt = \mu x$

Mean generation time (MGT) = $\ln 2/\mu$.

Such data provide (see Tables 3.1 - 3.2) empirical evidence that glucose levels do have an effect on growth rate. The first experiments involved growing cells at 10g/l-1g/l glucose and at a lower agitation rate (125rpm). This appeared to allow cells to grow at a quicker rate compared with agitation rates of 150rpm. This quicker rate of growth in the first experiment with 1g/l glucose may be due to a predomination of over respiration.

Table 3.1. Growth kinetics of glucose-limited baker's yeast at 125rpm agitation

Glucose(g/l)	1	2	2.5	5	10 + MN	10
Specific growth rate (h^{-1})	0.065	0.153	0.092	0.14	0.135	0.152
MGT(h)	10.67	4.53	7.53	4.95	5.13	4.56

(MN = micronutrients).

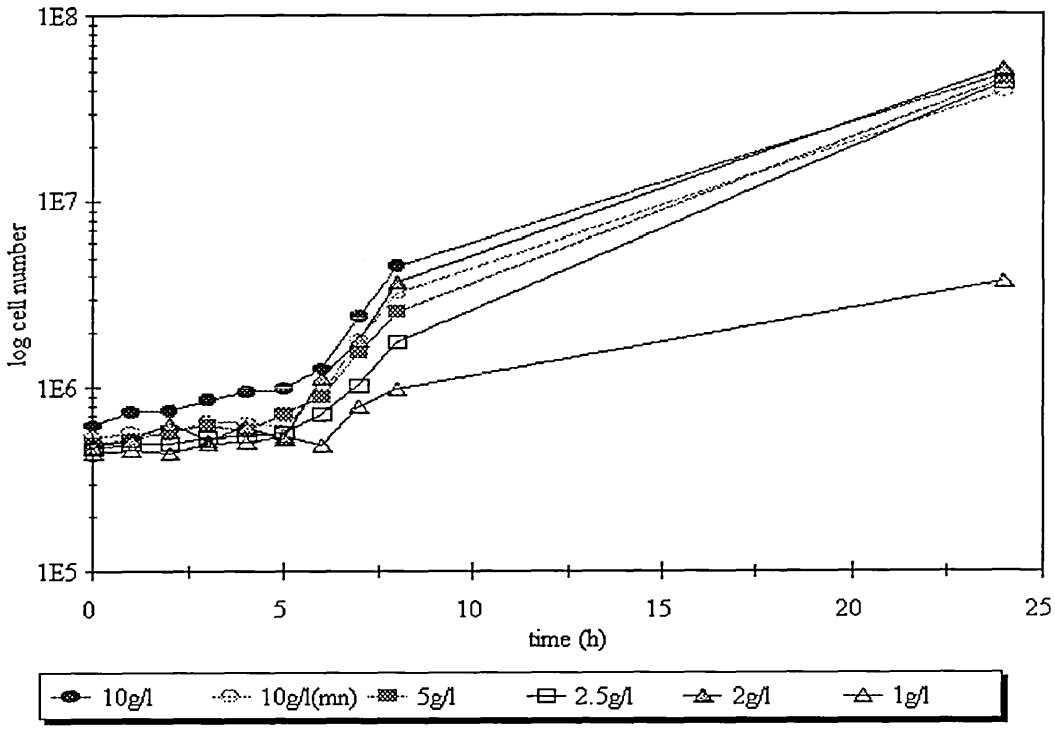
Table 3.2. Growth kinetics of glucose-limited baker's yeast at 150rpm agitation

Glucose(g/l)	0.1	0.25	0.5	1	1+MN	2
Specific growth rate (h^{-1})	0.003	0.004	0.011	0.034	0.034	0.074
MGT(h)	231	173.3	63	20.4	20.4	9.36

(MN = micronutrients)

Figure 3.1 Influence of different glucose concentrations on biomass and growth rate of baker's yeast in batch culture agitated at 125rpm.(mn = micronutrients)

a) Influence on biomass accumulation



b) Influence on growth rate

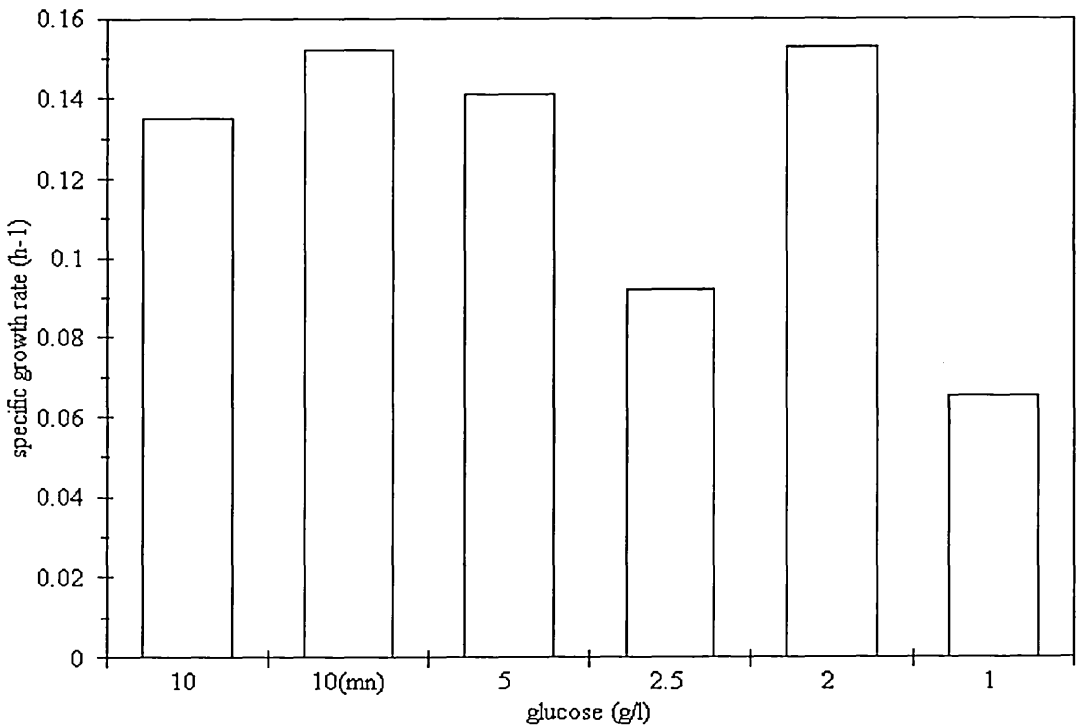
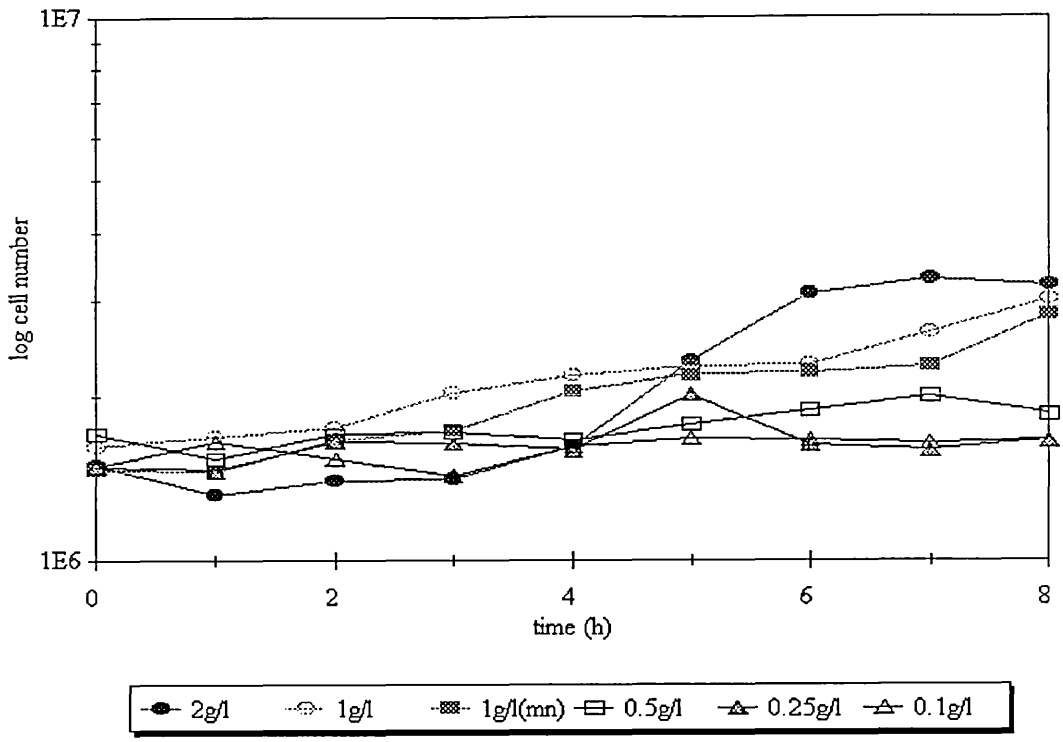
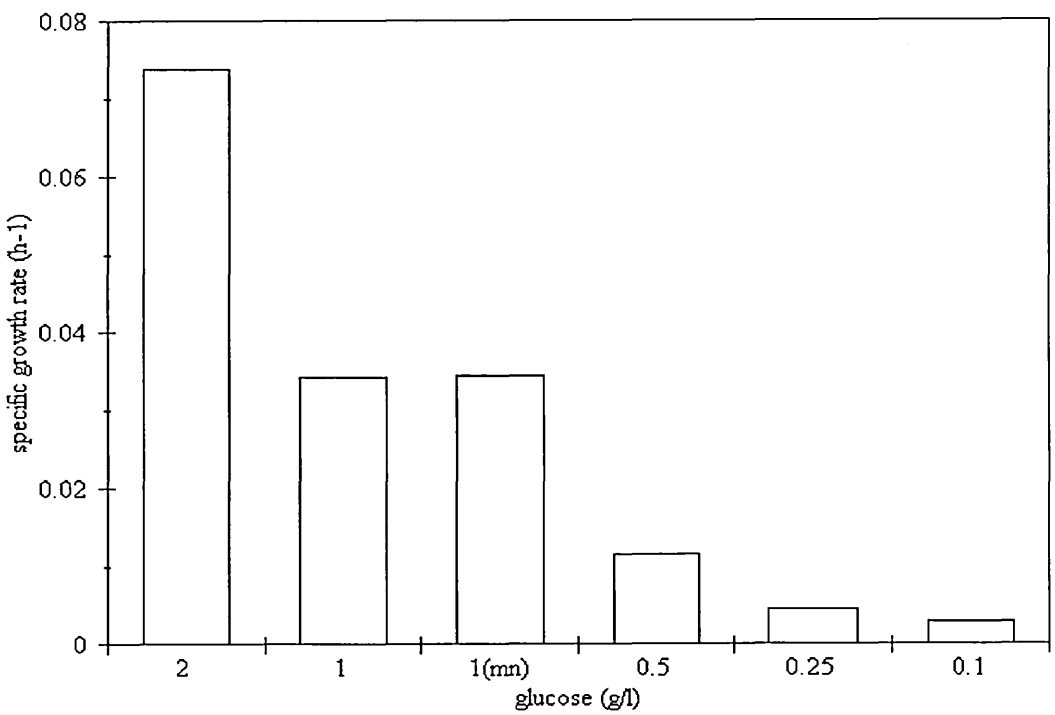


Figure 3.2 Influence of different glucose concentrations on biomass and growth rate of baker's yeast in batch culture agitated at 125rpm.(mn = micronutrients)

a) Influence on biomass accumulation



b) Influence on growth rate



3.3. Glucose effect on cell volume

An interesting observation arose during the glucose limitation studies that warranted further investigation. It appeared that under conditions of increasing glucose concentration, cell volume similarly increased. Previous research (Visser *et al.* 1995) suggested that mitochondrial morphology was related to aerobic or anaerobic metabolism, mainly through diffusion and condensing of the mitochondria, which altered the size of these organelles. This lead to an investigation into the effect of carbon source on cell size and in particular, the change, if any, in cell size due to the use of the carbon source by fermentation or respiration. For this, similar conditions were applied to the yeast in baffled shake-flask cultures as was carried out for the glucose-limiting experiments. The difference in this experiment was that a more sensitive technique was employed to measure ethanol in the cultures. This was achieved by using the HPLC described in Chapter 2 and by also growing the yeast in 2% ethanol as carbon-source, as ethanol can only be used as a respiratory carbon-source (i.e. ethanol is non-fermentable).

3.3.1. Results and discussion

Figure 3.3 shows that cell number increases as glucose concentration increases. It is apparent that as glucose increases then so too does the ability of the yeast culture to grow. The poorest growth is seen in 0.5g/l glucose and the best is seen in both 4g/l and 8g/l suggesting that an optimal growth rate is achieved under these conditions. This is further alluded to once growth rate is calculated (see Table 3.3. and Figure 3.4). Figure 3.4. also shows ethanol levels produced by each of these cultures and in this case the use of HPLC analysis for ethanol allows for the more accurate detection of ethanol in the 1g glucose/l culture and for the absolute absence of ethanol in the 0.5g/l culture. The

production of ethanol in the culture at 1g/l glucose level is most probably due to the imperfect aeration achieved. The consistency of alcohol production at all glucose concentrations is apparent with each producing a considerable amount of ethanol on the glucose they were supplied with.

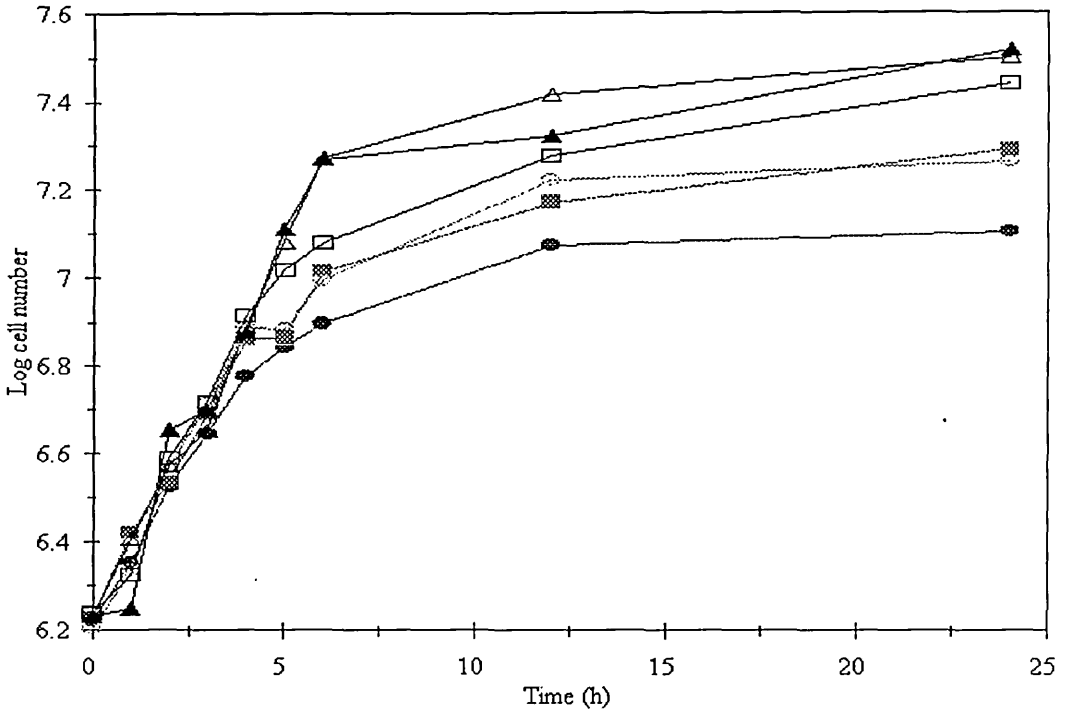
Glucose-dependent changes in cell size are shown in Figure 3.3. Yeast mean cell volumes consistently changed with increasing glucose concentration, since each rise in glucose resulted in a concomitant increase in mean cell volume. Unfortunately, due to the fact that all but those cells cultured in 0.5g/l glucose fermented (figure 3.4), it was difficult to determine whether the effect was due to glucose or the predominating catabolic fate of glucose.

Table 3.3. Growth kinetics of glucose-limited baker's yeast at 150rpm agitation with a different series of glucose concentrations

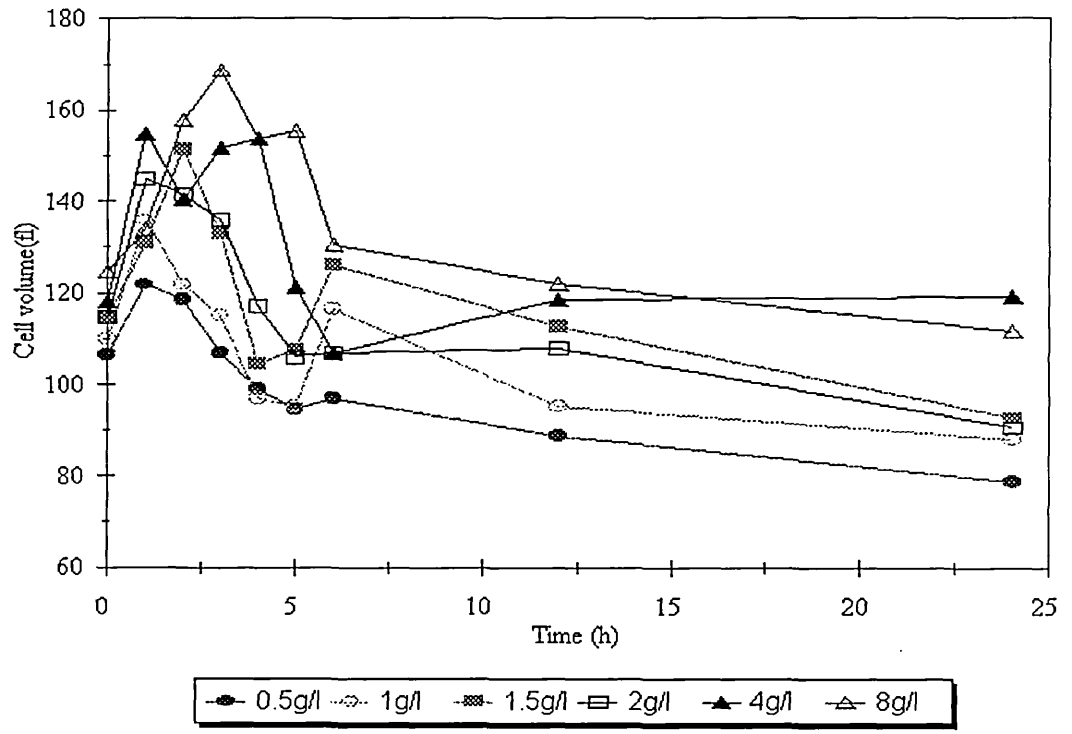
Glucose(g/l)	0.5	1	1.5	2	4	8
Specific growth rates (h^{-1})	0.06	0.055	0.077	0.083	0.197	0.191
MGT's(h)	11.55	12.6	9	8.35	3.52	3.63

Figure 3.3 Influence of differences in glucose availability on growth and cell size of baker's yeast in batch culture.

a) Influence on biomass accumulation



b) Influence on mean cell volume



Due to the inability to determine whether or not the change in cell size seen in Figure 3.3 was due to glucose or it's predominant catabolic fate, it was deemed necessary to study the same parameters in cultures growing on a non-fermentable carbon-source. The results of such an experiment, shown in Figure 3.5, clearly showed that there was no change in cell volume when the cells were grown with 2% v/v ethanol as carbon-source, whereas cell size more than doubled when the cells were grown on 2% w/v glucose. Interestingly, cells grown in two other glucose concentrations used exhibited fermentative behaviour in the first 12h that also coincided with the cells increasing their volume. Noticeably the cells cultured in 5g/l glucose did not have any ethanol present after 24h and this coincided with the culture mean cell volume returning to their size upon inoculation. The 10g/l and 20g/l cultures both finished with elevated cell volumes and this was consistent with them having undertaken fermentative metabolism. Both these cultures and the 5g/l culture showed a decrease in cell volume after 12h, but in the case of the 10g/l and 20g/l cultures, there was a high residual ethanol level in the culture. This may be the reason for the decrease in cell volume. Measuring the glucose levels in the medium throughout the experiment would have given a useful indicator of what these cells were using as a carbon source after 24hr. Similarly, allowing all of the cultures more time to assimilate their respective C-source would also have allowed a more affirmative statement about the reason for the increase in cell size. Current evidence suggests that the increase in cell volume exhibited is a function of C-source and metabolic fate of that source. Oxygen levels were not measured but the results from 2% ethanol and 5g/l glucose cultures indicated that the experimental conditions were not anaerobic and thus the changes seen in cell volume were not due to presence or absence of molecular oxygen.

Perhaps the Crabtree effect in *S. cerevisiae* exists to confer an environmental advantage on this organism, as it preferentially ferments sugars to ethanol and then utilises this ethanol. The ability to increase cell volume would serve to increase surface area/sugar contact, thereby allowing greater diffusion or membrane transport of exogenous sugar into the cells and increasing the cells ability to produce ethanol at a faster rate. Once available glucose has been fermented, or availability of glucose is reduced to the point that ethanol can be consumed, then the need for larger cell sizes may not be necessary.

Figure 3.4 Influence of glucose concentration on baker's yeast growth rate and ethanol production.

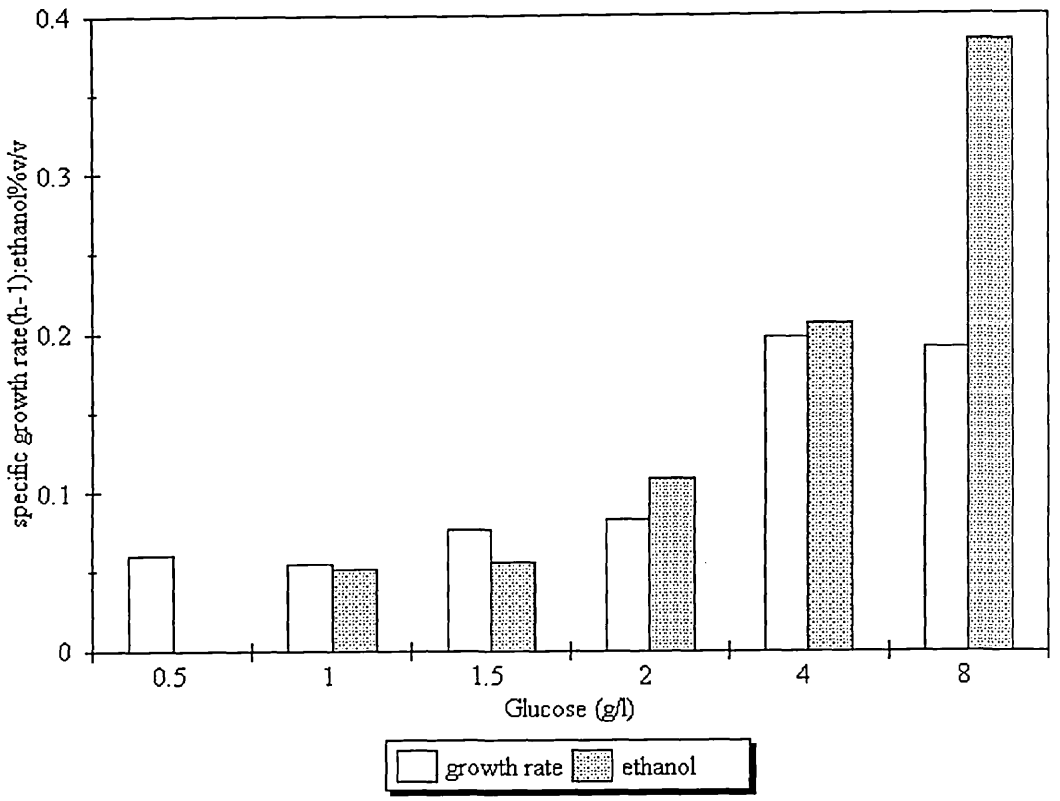
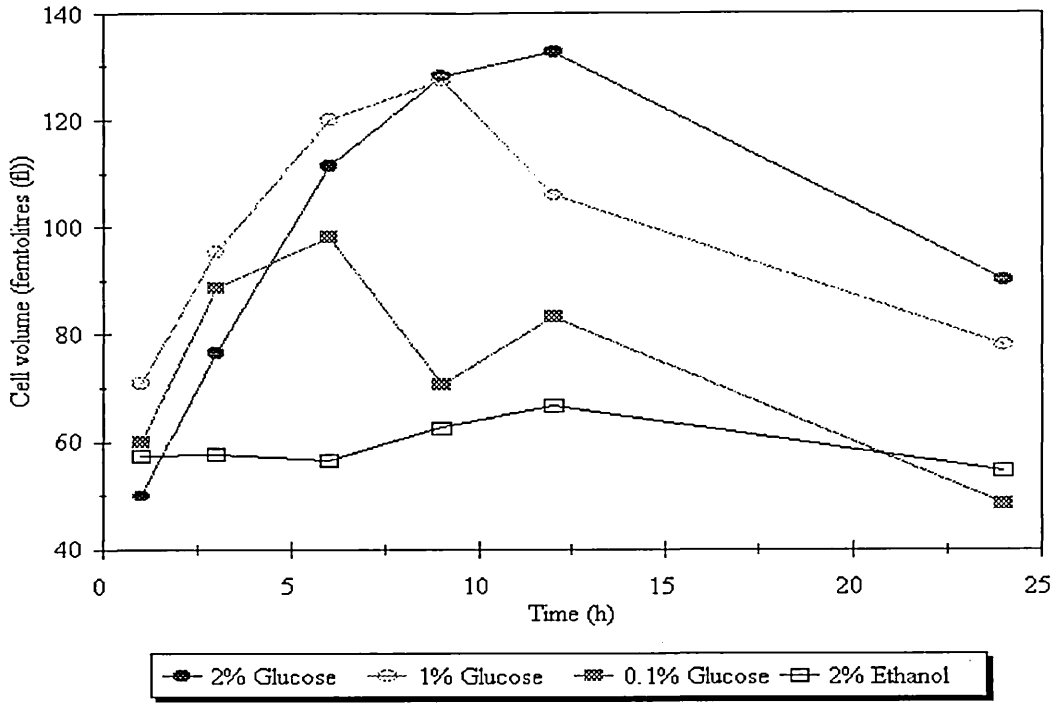
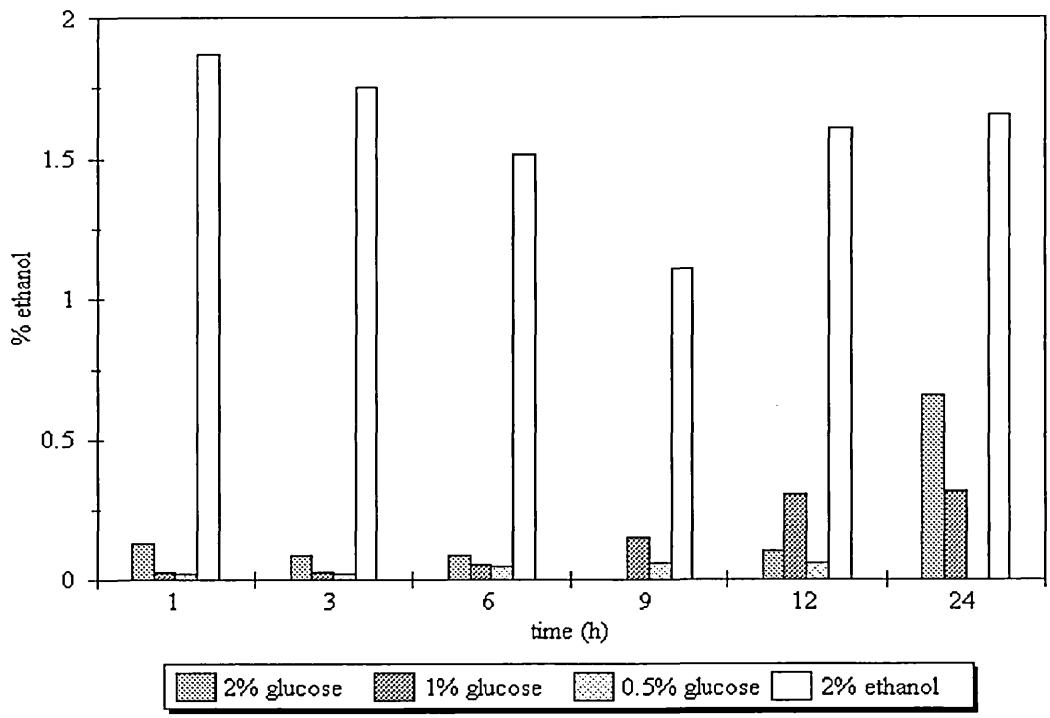


Figure 3.5 Influence of carbon-source on yeast mean cell volume and metabolism.

a) Influence on mean cell volume



b) Influence on yeast cell metabolism.



3.4. Glucose-limited yeast physiology in continuous culture

Once the glucose limiting concentration had been determined, it was possible to design a chemostat that would be used for the experiments to study yeast physiology under steady-state conditions. The whole concept of a chemostat requires the practice of constantly reviewing the operating system and it's parameters and deciding on whether there is a need to add another device or improve on an existing one. The chemostat utilised in this research was continually developed and refined. For example, it was originally hoped to have on-line measurement of cell growth using a spectrophotometer with a flow-through cuvette. This would have allowed calibration of the culture density with dry weight and the use of a chart recorder to account for changes in dry weight during cultural growth. Though a few trial runs were successfully carried out using this device it did not allow the use of pH control (lack of suitable vessel ports). This was one of the reasons for deciding not to control the pH of the chemostat. Two other reasons for this was that, i) allowing the yeast to reduce the pH would give them an advantage over most probable contaminants and ii) the actual reduction in starting pH from 4.5 to 3.2 is not believed to affect the yeast's physiology or metabolism (Madaree *et al.* 1991; Angelov *et al.* 1997), although there are conflicting reports on which pH range is most suitable for fermentation (Sengupta and Sadhukhan 1992; Parsons *et al.* 1984). Another major reason for finally abandoning the on-line turbidity measurement was the apparent adherence of the yeasts to the flow-through cuvette, which resulted in a number of technical problems (i.e. biofilm development) that could not be solved adequately .

Once the chemostat was constructed, various cultures were propagated in order to produce samples of yeast grown at specific growth rates. For these experiments, samples were collected in medicine flat bottles through the sample device described in Chapter 2, section 2.4. This meant that for each sample a volume of 300ml of yeast culture was removed from the vessel, once steady state had been established, and used for analysis. This amount of volume loss from a chemostat is known to considerably upset the steady state conditions in the vessel and also have a considerable knock-on effect on the cells growth rate. The chemostat therefore required a period of time to re-establish itself as continuous with a further period of time to return to a steady state where a duplicate sample for the cells at that growth rate could be obtained. The times required for each of these conditions varied according to the delivery rate of the medium feed pump, and it is appropriate to refer to these periods as "vessel volume changes". This allowed for a constant parameter to be attached to each growth/dilution rate at which the steady-state of the culture should have occurred. In general, a minimum of 6 vessel volume changes was considered sufficient for re-establishment of steady-state. As a rule none of the continuous cultures were considered steady state in this research until at least 6 vessel volume changes had occurred and generally in this set of experiments 8 vessel volume changes was the standard applied.

The growth rates selected for study were largely dictated by the feed pump rates. The tubing used to supply the medium was altered to provide a wider range of dilution rates and therefore, growth rates. The range of growth rates that were available for study using the chemostat were sufficient for providing a detailed profile of how baker's yeast performs under conditions that were to be referred to as "normal". These

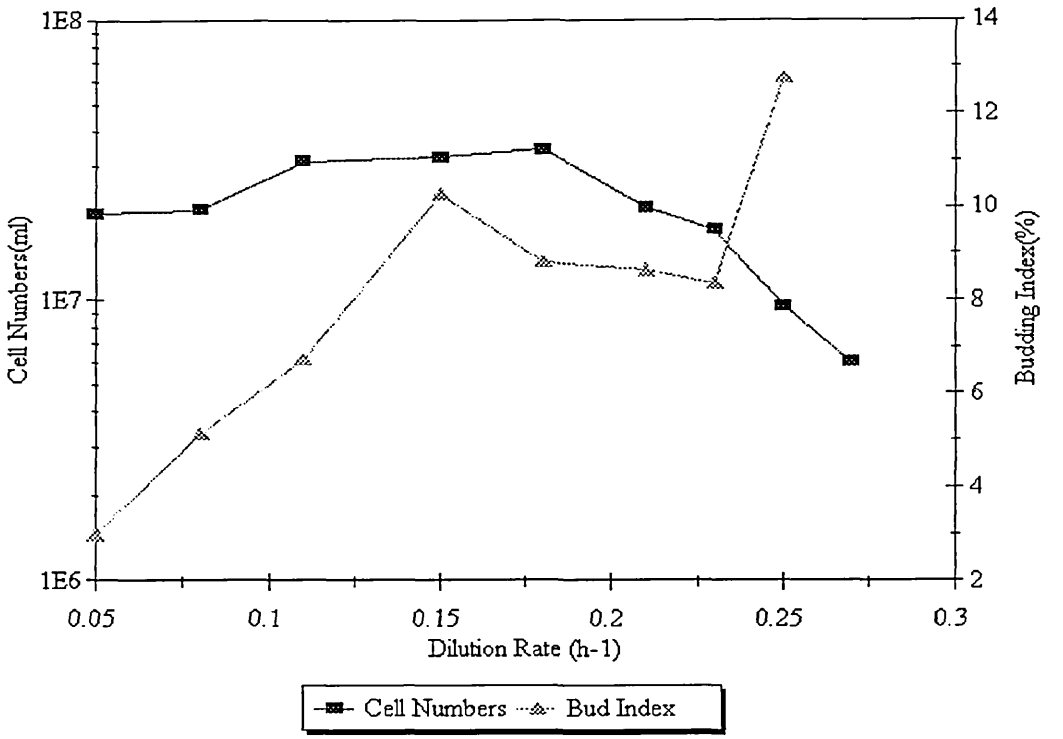
conditions remained constant throughout the chemostat experiments. The temperature was maintained consistently at 30°C ± 0.1°C, agitation at 300rpm, aeration at 3.5VVM and pH allowed to fall freely as the yeasts acidified the medium. The pH started at 4.5 and fell to around 3.2-3.3 for every run. Failure of the culture pH to fall as low as 3.4 was generally a good indicator of contamination within the vessel early on in its operation.

3.4.1 Results and Discussion

The results, presented in Figure 3.6, show that as dilution rate/ specific growth rate increases then so to does the budding index. There is little change through increase in specific growth rate in cell numbers and this is reflected in the dry weight results presented in Figure 3.7. As can be seen from Figure 3.6 cell numbers start to decrease just before a growth rate of $0.2h^{-1}$, but dry weight is not affected until a growth rate closer to $0.24h^{-1}$ was reached. Interestingly, the budding index increased quite considerably as the biomass declined and this may be due to the increasing availability of glucose, or to the switching in metabolism from respiratory to respiro-fermentative. These results are consistent with steady state cultures in that biomass levels are not affected by the change in growth rate but the physiology of the cells may be altering during these changes. It is known that in *S. cerevisiae* there are a few distinct physiological states that can exist in steady state that are dependent on the rate at which the culture is growing (Munch *et al.* 1992; Duboc *et al.* 1996).

Figure 3.6 Effect of dilution rate on the growth and physiology of baker's yeast grown in a chemostat at 30°C

a) Effect on cell number and budding index



b) Effect on intracellular levels of trehalose and protein

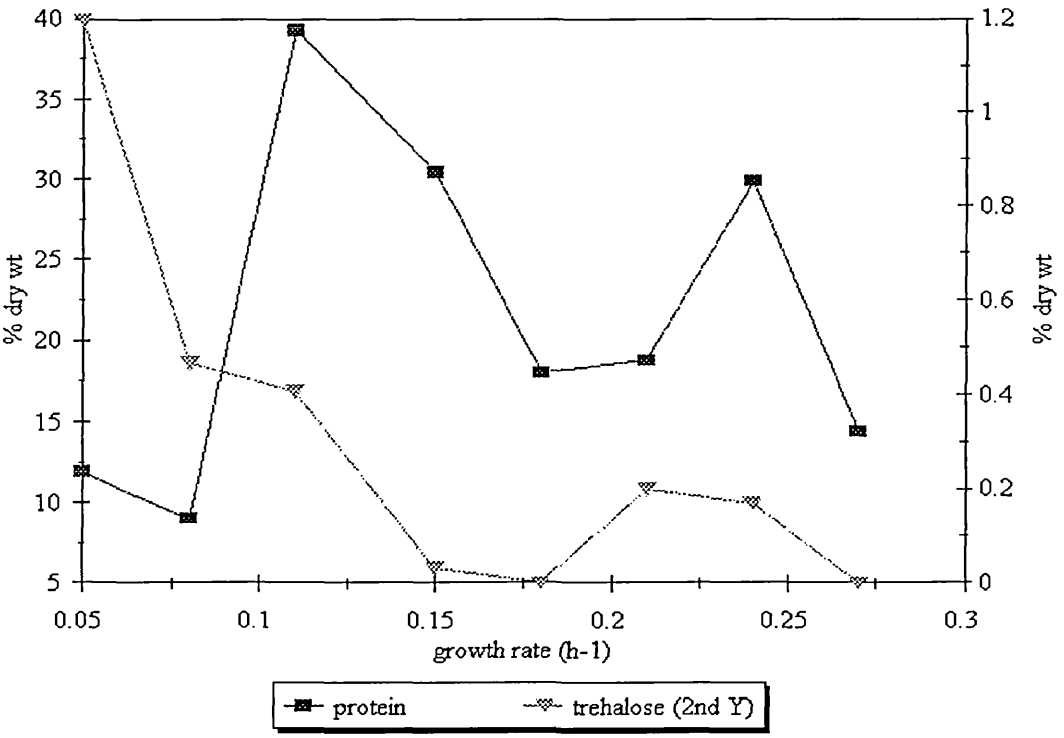
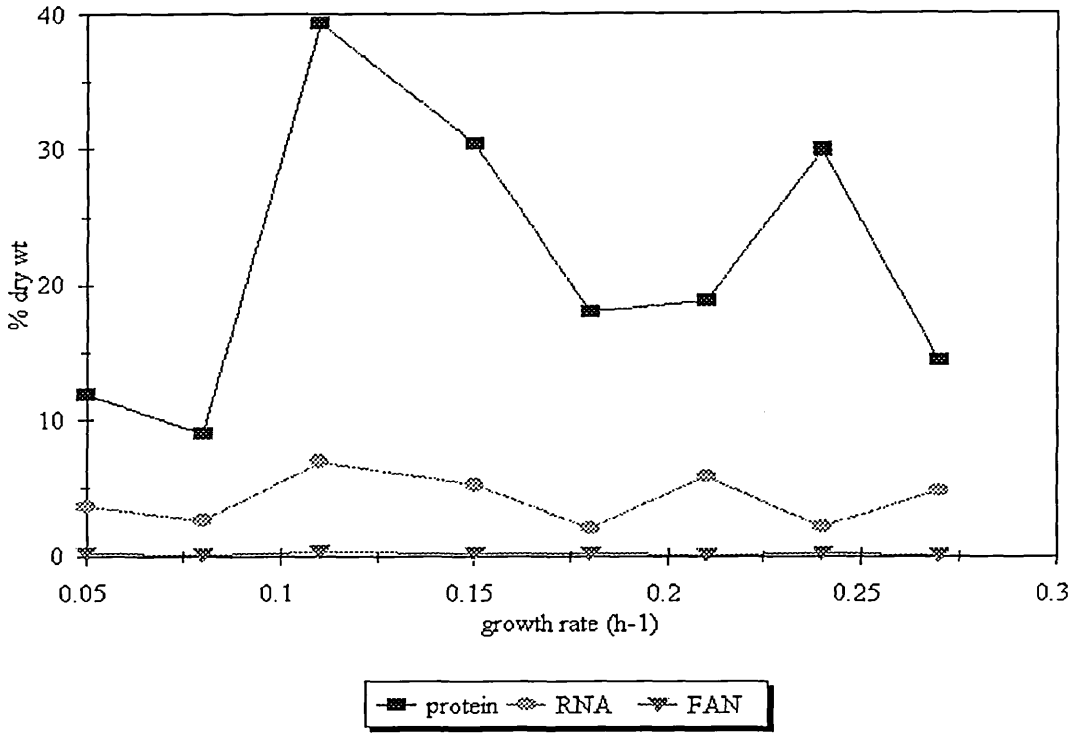
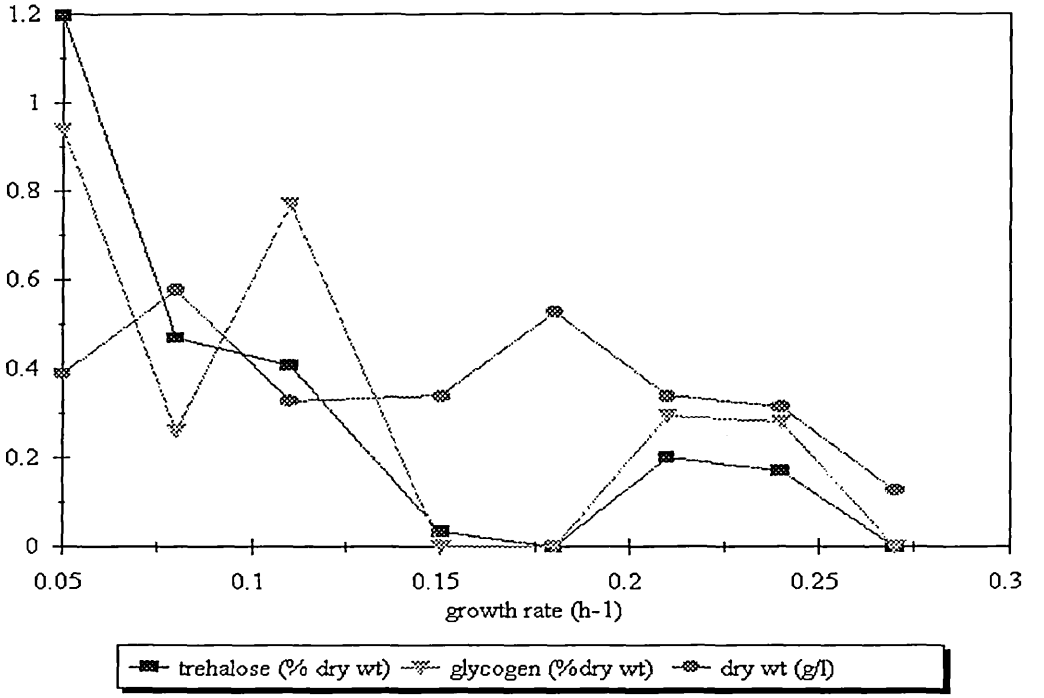


Figure 3.7 Effect of dilution rate on intracellular macromolecular composition of baker's yeast cultivated in a chemostat at 30°C

a) Effect on the major nitrogen compounds protein, FAN, and RNA



b) Effect of dilution rate on biomass and major carbohydrates trehalose and glycogen



Separately, the effects of dilution rate on the intracellular components are also shown in Figures 3.6 and 3.7 with the effects on protein and trehalose content being compared with each other and as part of the intracellular components to which they belong, e.g. DNA/RNA/protein metabolism and storage carbohydrate metabolism. There were many reasons for looking at trehalose in relation to the protein content of the yeast. The main area of interest in cellular trehalose and protein to yeast production is that, depending on the final product, there is a preference to which of these is more desirable. Increased trehalose, for example, can result in cells adapting to the stresses involved in the autolysis process and cause changes in the final extract produced.

The results for cell protein in Figures 3.6 and 3.7 show that protein content rises from around 10% of the dry cell weight at low dilution rates to 40% dry cell weight (DCW) at $0.11h^{-1}$, before falling back to about 20% DCW at dilution rates close to the optimal rate ($0.18h^{-1}$) for this yeast. There was little effect of change of dilution rate on the levels of RNA and FAN in the cells. This may be due to the rapidity at which the cells turnover RNA and proteins during their metabolism. Rises in levels of RNA would indicate much greater levels of transcription while also suggesting a slower rate of RNA turnover. This does not appear to be the case in these cells. Changes in the cellular levels of FAN would indicate greater levels of protein turnover at the expense of protein biosynthesis. Again, this does not appear to be the case, even when the cells enter the washout phase of growth at the critical dilution rate ($D_c = 0.27h^{-1}$). FAN levels would have been more informative had the level of self-autolysis of the culture been investigated, since as the number of cells autolysing increases, then so too should the level of FAN. The rise seen as cells pass through low dilution rates to sub-optimal

dilution rates may be due to the cells moving from a state in which maintaining biomass was less expensive in terms of cell energy utilisation, as can be seen from the accumulation of both trehalose and glycogen in cells at lower dilution rates. At the switch in dilution rates it would appear that accumulation of both these carbohydrates is reduced as the cells require more of the limited supply of glucose to maintain a biomass that is capable of growing at the increased dilution rates. Protein levels rose again after $0.22h^{-1}$, which may be consistent with the cells passing their critical dilution rate at which glucose could no longer be metabolised solely by respiration. Under such conditions the enzymes involved glycolysis are known to increase in proportion and during fermentation can account for as much as 70% of the cell's total protein (Zimmerman and Entian 1998).

This may also be supported by the re-accumulation of both trehalose and glycogen in the cells after the optimal growth rate for respiratory growth is surpassed, which is consistent with the behaviour of yeast cells during growth on glucose (Thevelein 1996; Wiemken 1990), although trehalose is not normally accumulated until the onset of stationary phase. Changes in growth rate have previously been recorded as effecting the levels of trehalose within such cells, and are normally related to stresses exerted upon these cells (Arneborg *et al.* 1995). The accumulation of trehalose at the onset of stationary phase cannot be the reason for the presence of this carbohydrate in these cells. It's presence at levels of about 0.2% DCW suggests that although the cells are not stressed they may not be growing at their physiological optimum. Some research has been carried out to determine whether or not the cellular levels of trehalose or glycogen are useful indicators of brewer's yeast physiology (O'Connor-Cox *et al.* 1996b). In

brewing, it appears that both are good indicators of the status of cells before use in fermentation. It may be that the detection of both carbohydrates in cells grown at sub- and supra-optimal conditions in chemostat cultures may be a meaningful indicator of their physiological condition.

3.5. Chemostat phenomena

As the time over which the chemostats ran became longer and longer, it became apparent that the yeast cells within the culture started to behave unusually, particularly in their ability to adhere to surfaces within the vessel. This led to an interest in what may be happening to the yeast cells in the culture during prolonged periods of cultivation. Another phenomenon that had been reported in a number of publications was the synchronisation of cell division that could occur within a culture growing at low dilution rates in chemostats (Abel *et al.* 1994; Parulekar *et al.* 1986; Keulers *et al.* 1996; Satroutdinov *et al.* 1992).

During the running of the six chemostats that provided the results discussed in section 3.3, results were also gathered for cell numbers, bud index and mean cell volume. Only the cell numbers and bud index were presented as they represent useful indicators of the state of the cells at any given dilution rate, with respect to biomass levels. In the analysis of cell numbers, bud index and mean cell volumes are given to indicate the continuity of the yeast culture through time.

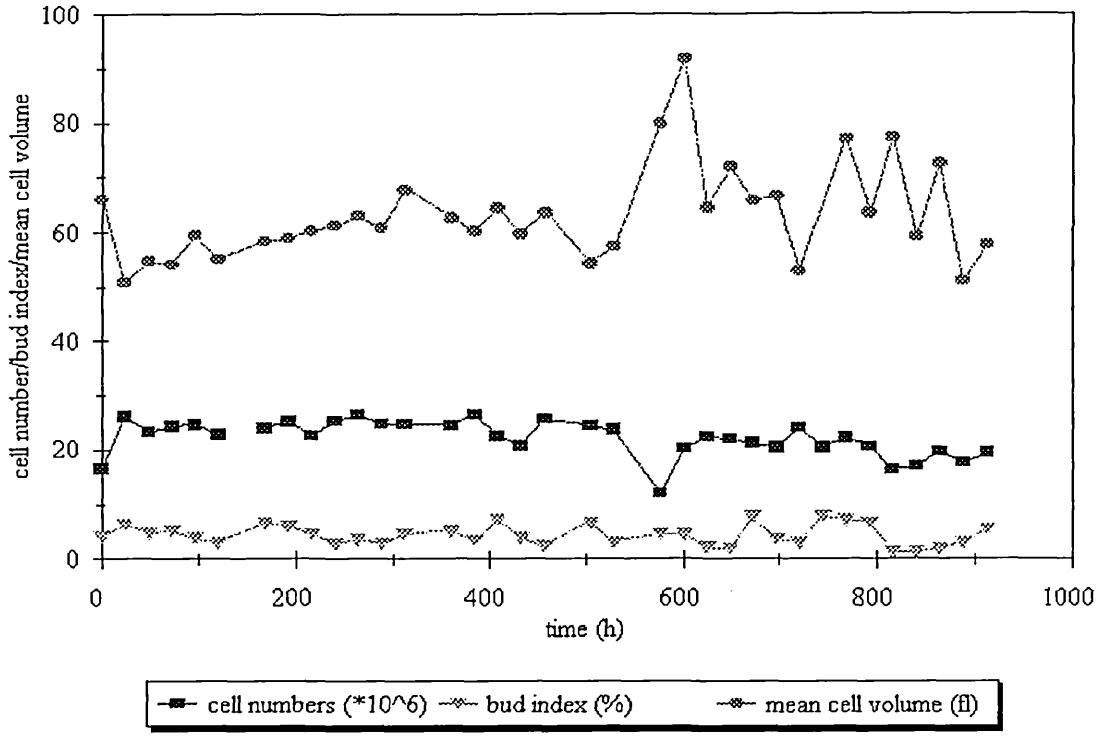
3.5.1. Results and Discussion

3.5.1.1. Longevity

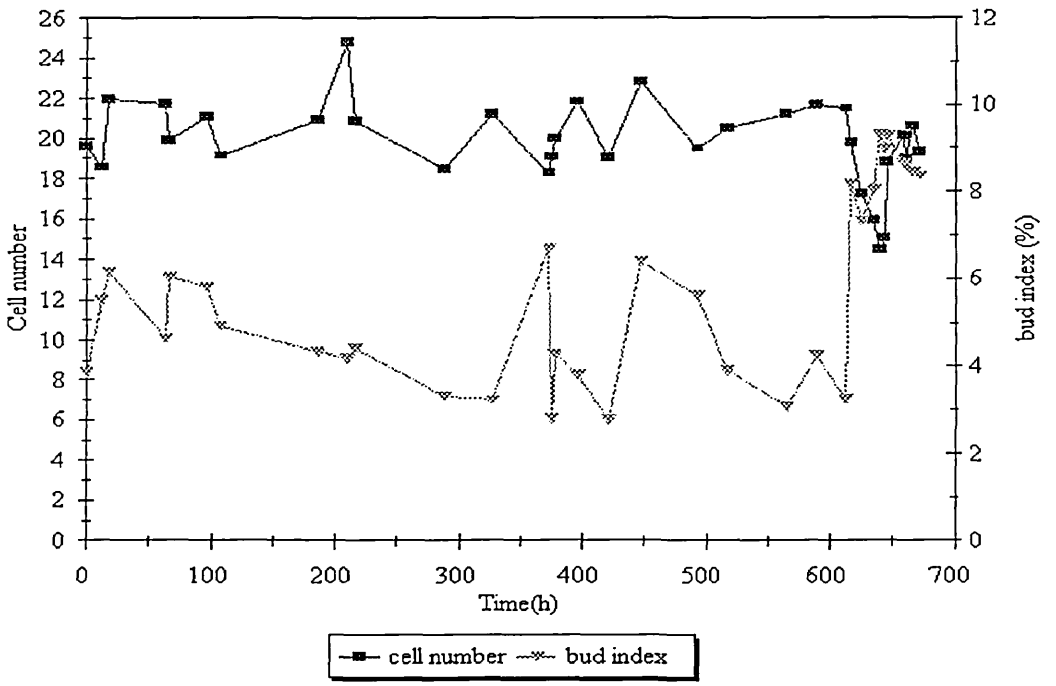
Figure 3.8 shows the changes in cell numbers and bud index in two different types of chemostat cultures. The difference between these two cultures is that in Figure 3.8a the propagation was halted due to the yeast adhering to the vessel components and even growing in the feed tubing. Figure 3.8b shows the response of the yeast when the propagation was halted due to contamination by another organism (identified, preliminarily as a *Fusarium* sp.). Contamination of the chemostat by a fungal species only appeared after a prolonged propagation where air filters were not routinely replaced with sterile new filters. In cultures where other contaminants were identified, (e.g. *Rhodotorula* sp.) the same response was recorded in the yeast, that is an initial decrease in cell number accompanied by a rise in bud index. In the case of *Fusarium* contamination, *S. cerevisiae* seemed capable of adapting to the new environment, despite the obvious competition for the limited amount of glucose.

Figure 3.8 Effect of prolonged chemostat culture on the biomass and cell size of baker's yeast

a) Response of cells to prolonged cultivation



b) Response by a culture of baker's yeast to a contaminant organism (observed from 600h onwards)

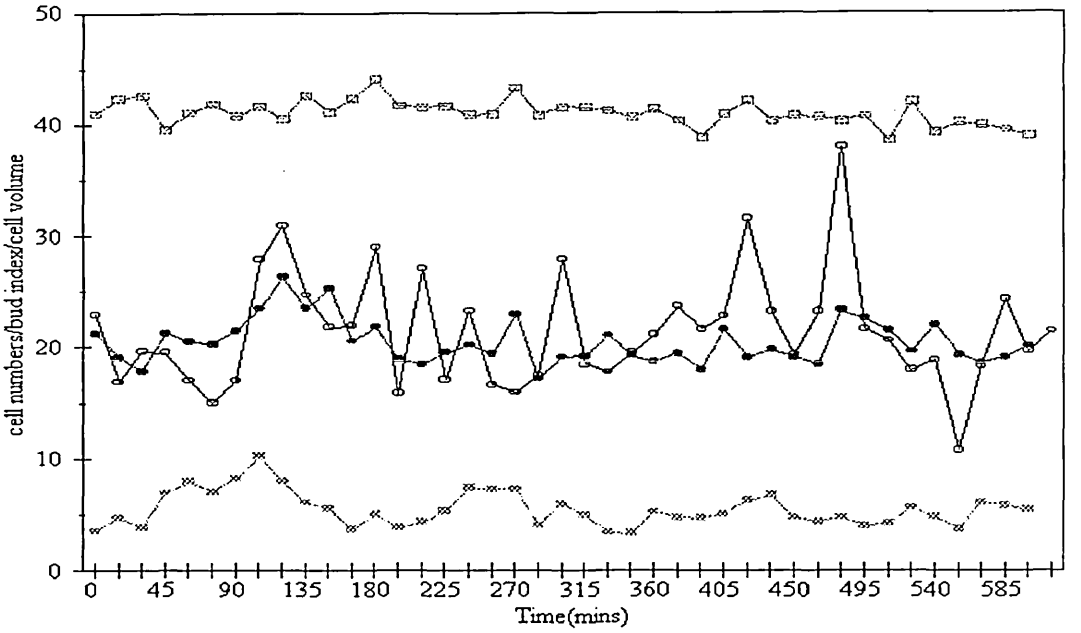


3.5.1.2. Synchronicity

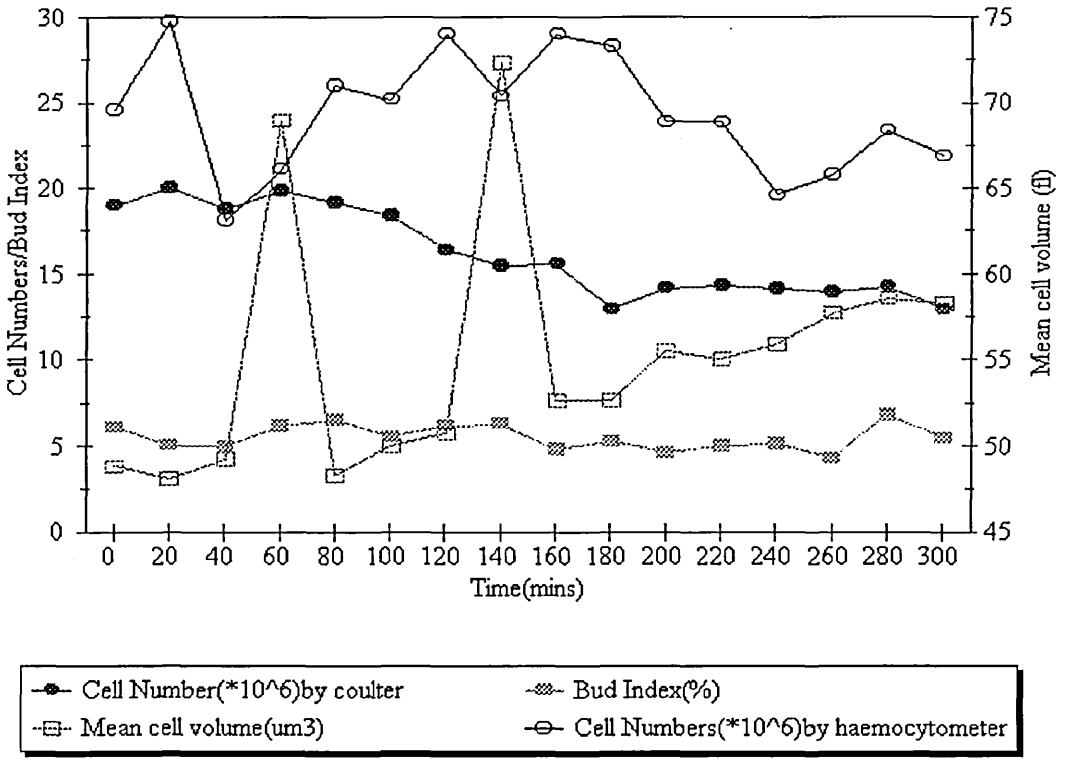
This phenomenon is characterised as a concerted period of cell division by the majority of the population of the cells at lower dilution rates. In Figures 3.9, it was clear that at both dilution rates studied, no great synchrony in either of the cultures was observed. Bud index can be useful for determining the degree of synchrony in a culture, as the majority of cells (70% in some cases) will be budding at the same time. It was interesting to note that the cell numbers were more consistent when measured by Coulter counter rather than by haemocytometer. It can also be seen that the cells growing at $0.1h^{-1}$ that both cell number estimations were quite closely related with only the inconsistency of the haemocytometer method giving rise to the larger variations. The cell numbers in the culture growing at the faster dilution rate were 20% higher when estimated by haemocytometer compared with Coulter counter. Difference in results while maintaining a similar cell number can easily be explained away through experimental error, as in the culture at $0.1h^{-1}$, but it is more difficult to explain such a large variance between the two methods at higher dilution rates. One of the possible explanations for these differences will almost certainly be due to the inability of the coulter counter to count two cells when heamocytometry would reveal two cells, due to the size of the attached state of a daughter cell that may no longer be counted, manually, as a bud.

Figure 3.9 Incidence of synchrony in the biomass of a chemostat cultures of baker's yeast

a) Synchrony in baker's yeast at a growth rate of $0.10h^{-1}$



b) Synchrony in baker's yeast at a growth rate of $0.20h^{-1}$



3.6 Summary

In this Chapter a few facts have been elucidated about an industrial strain of baker's yeast. The first of these is that 1g/l glucose is sufficient to allow cell growth through respiration but not allow the onset of fermentation under aerobic conditions. From the data presented here it is also possible to show that the metabolism of *S. cerevisiae* has an important bearing on the cell volume, with cells that are respiratory having a much smaller mean cell volume. This effect is seen to relate to the amount of fermentable glucose the cells are exposed to, i.e. greater amounts of glucose results in a greater increase in their mean cell volume.

In steady-state chemostat cultures it was evident that the growth rate was influential on the macromolecular composition of the yeast. The trends recorded here are consistent with previously reported results (Ertugay and Hamamci 1997). There appeared to be no evidence of synchronicity in the yeast culture at either low (0.1h-1) or high (0.2h-1) growth rates. This could be due to the lack of a particular effector of synchronous culture, many of which are believed to be due to be metabolic in nature, especially with regard to fermentative metabolism (e.g. ethanol, CO_2). This is believed to be due to the forced change of growth rate in individual cells at the onset of budding (Munch *et al.* 1992).

CHAPTER 4 - YEAST PHYSIOLOGY IN RESPONSE TO GLUCOSE AVAILABILITY : EXPRESSION OF THE CRABTREE EFFECT

4.1. Introduction

The control of industrial propagations of *S. cerevisiae* ensures that molasses is fed to the yeast cells at a rate that keeps the sugar concentration below 0.1%w/v. This keeps the cells glucose-limited, which is paramount in efficient production of biomass from *S. cerevisiae*. Threshold glucose concentrations which repress respiration in *S. cerevisiae* are strain dependent. Above these thresholds, fermentative metabolism will result which causes a concomitant, and expensive, loss in biomass.

Changing glucose availability during chemostat culture would, of course, affect the glucose-limited state of the cells, but it would also allow a study of the way yeasts respond to this event. Such studies are highly relevant to industrial yeast propagation processes. Comparative studies with yeast species that possess different glucose sensitivities were deemed valuable in this regard. For example, by comparing a Crabtree positive yeast (*S. cerevisiae*) with a Crabtree negative yeast (*Kluyveromyces marxianus*) in chemostat cultures when glucose becomes non-limiting, insight may be gained into various aspects of metabolic regulation which are relevant to yeast biotechnology.

As discussed in Chapter 1, the Crabtree effect along with that of glucose repression, is of major economic significance. It is now widely recognised that *S. cerevisiae*, due to it's Crabtree positive behaviour is an organism that is not ideal for the

production of biomass on an industrial scale. Other yeasts are now being exploited in this regard, particularly in high cell density fermentations aimed at production of recombinant proteins (Mendoza-Vega *et al.* 1994). *Kluyveromyces* sp. have two advantages in relation to industrial yeast biomass production: i) they are classed as Crabtree negative, ii) some species are capable of using lactose as a carbon source and thus able to utilise waste residues from the dairy industry.

Significantly, both *S. cerevisiae* and *K. marxianus* are already in use in different industrial applications and both have Generally Regarded as Safe (GRaS) status, allowing them to be used as food or fodder agents. The ability of *K. marxianus* to respire even in high levels of sugar suggests that it would be of greater application in biomass production. Another advantage that some strains of *K. marxianus* have is their ability to grow at higher temperatures (e.g. over 45°C), which may prove more economical in regions where cooling may be more problematical. Other *Kluyveromyces* sp. also have these same thermotolerant characteristics.

4.2. Experimental approach

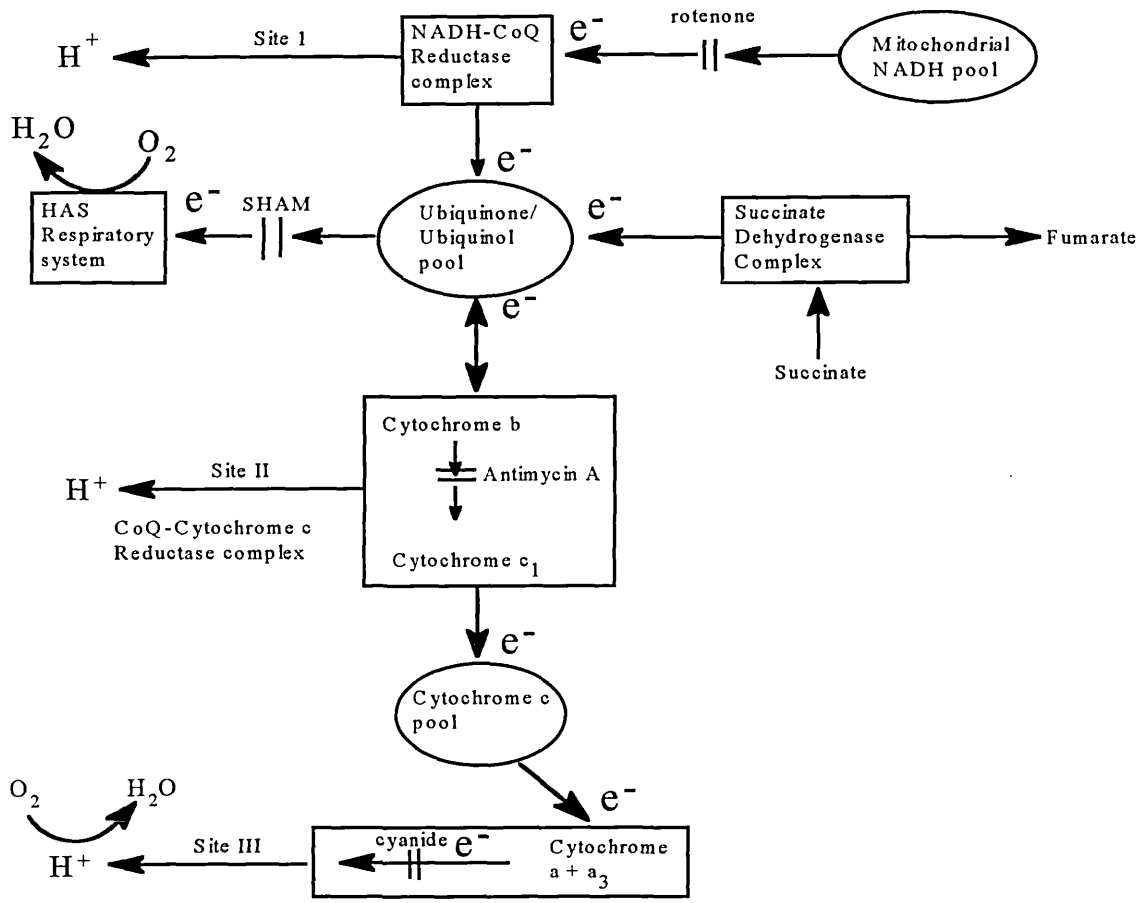
The chemostat described in Chapter 2 was used in the following experiments with alterations in the glucose concentration of the feed medium. In each case the glucose level was changed from 1g/l to 50g/l and allowed to run at this glucose concentration for 24h where upon the reservoir was changed back to glucose at 1g/l. This routine was carried out on two separate cultures of each of the yeast species studied, the results presented here are averages of the responses of the yeasts in the duplicate cultures. As

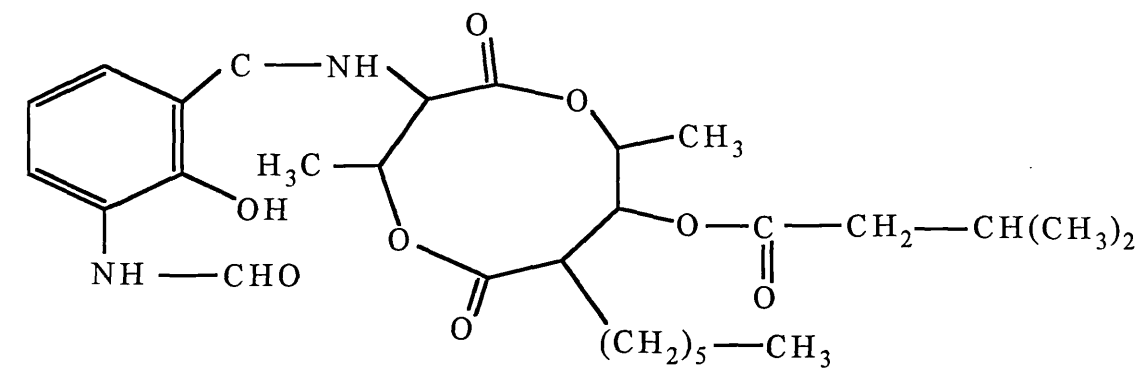
part of the design of these experiments, a few important assumptions were made that affected the sample regime for the two yeast species that were studied. In essence, glucose repression phenomena in *S. cerevisiae* are extremely well known and therefore, for *S. cerevisiae* it was necessary to sample for trends that were existent on some of the other parameters of interest. Ethanol production was expected to occur almost immediately on the change from 1g/l feed glucose to 50g/l feed glucose. In the case of *K. marxianus*, there was uncertainty as to how this yeast would react to the change in glucose feed concentration and so a much more thorough sample regime was implemented.

The uncertainty involved arose from the results of batch studies on this yeast and a selection of other yeast species that may or may not exhibit the Crabtree effect or fermentative metabolism. The work in these experiments involved growing the yeasts in yeast nitrogen base (YNB) (Difco corp. USA) at 1g/l glucose, 50g/l glucose and 50g/l glucose + antimycin A. Antimycin A (from *Streptomyces* sp.) was obtained from Sigma (Poole, UK) and prepared in ethanol. The final concentration added to yeast cultures was 5mg/l. In these experiments measurements of cell growth, ethanol production, oxygen uptake and excretion of metabolites from the cell were measured. Most of the methods used in these analyses are described fully in Chapter 2 except for the measurement of oxygen uptake. Oxygen uptake was measured as described by the method of Liti *et al.* (1999). Antimycin A acts by inhibiting the transfer of electrons from cytochrome b to cytochrome c (Figure 4.1). This allows a more definitive determination of the ability of the yeast to continue growing by switching metabolism to fermentation, or by expressing an alternative respiratory pathway (Alexander and Jeffries

1996); (Lodolo *et al.* 1999).

Figure 4.1. Diagram depicting electron transport chain and the structure of antimycin A.





Antimycin A

The investigation of the response of yeast species to antimycin A was thought to be important in determining those yeast species that were truly respiratory, respiro-fermentative or fermentative. The yeast strains that had no fermentative ability would have their growth completely halted if the concentration of antimycin A was high enough and they did not posses an alternative respiratory pathway. The yeast strains that were used in this study are shown in Table 4.1 and were supplied by Mr. G. Liti from the industrial yeast collection, DBVPG, University of Perugia, Italy. These yeasts were selected on their ability to respire or ferment glucose. Only *Wickerhamiella domercqiae* was described as being non-fermentative. What was unknown about some of the yeast species was their expression of a Crabtree effect. For this reason, known Crabtree positive yeast species were included, i.e. *S. cerevisiae* and *Schizosaccharomyces pombe*.

Table 4.1 Yeast species used in antimycin A studies.

Yeast species	Catalogue yeast determination	Crabtree effect
20 Castelli	*Saccharomyces cerevisiae* 20 Castelli	positive
GB4918	*Saccharomyces cerevisiae* GB4918	positive
DBVPG 6275	*Schizosaccharomyces pombe* DBVPG 6275.	positive
DBVPG 4124	*Candida stellata* DBVPG 4124.	negative
DBVPG 6167	*Torulaspora delbrueckii* DBVPG 6167.	negative
DBVPG 6305	*Kluyveromyces lactis* DBVPG 6305.	negative
DBVPG 6075	*Kluyveromyces lactis var. drosophilarum* DBVPG 6075.	negative
DBVPG 6108	*Kluyveromyces lactis var. phaseolosporus* DBVPG 6108.	negative
DBVPG 6112	*Kluyveromyces lactis var. vanudenii* DBVPG 6112.	negative
DBVPG 6165	*Kluyveromyces marxianus* DBVPG 6165.	negative
DBVPG 6775	*Eremothecium coryli* DBVPG 6775.	positive
DBVPG 6788	*Wickerhamiella domercqiae* DBVPG 6788.	negative non-fermentative

4.3. Results and Discussion

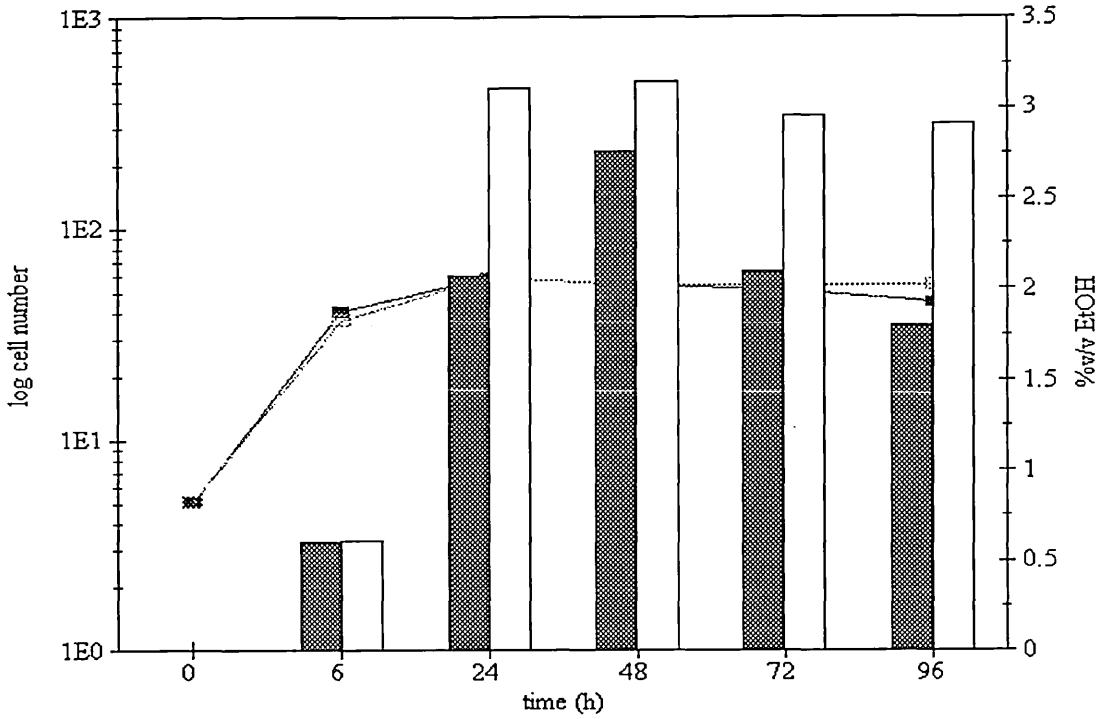
4.3.1. Growth of *S. cerevisiae* and other yeasts in the presence of antimycin A.

Prior to describing the behaviour of the yeast *S. cerevisiae* and *K. marxianus* in chemostat cultures subjected to alterations in glucose concentration (4.3.2), it was deemed necessary to briefly explain their respiratory behaviour in batch culture. This also involved studying their ability to ferment in the presence of a respiratory inhibitor, antimycin A. As is shown in Figure 4.2 there is no effect of antimycin A on the growth of *S. cerevisiae* but it is clear that the cells metabolism is altered as can be seen from the promotion of ethanol production. *K. marxianus* had its growth rate slowed by the presence of antimycin A but it was able to continue growth by increasing its rate of fermentation. By the end of the experiment, the level of growth in the presence of antimycin A was similar to that in those cells grown in the absence of antimycin A.

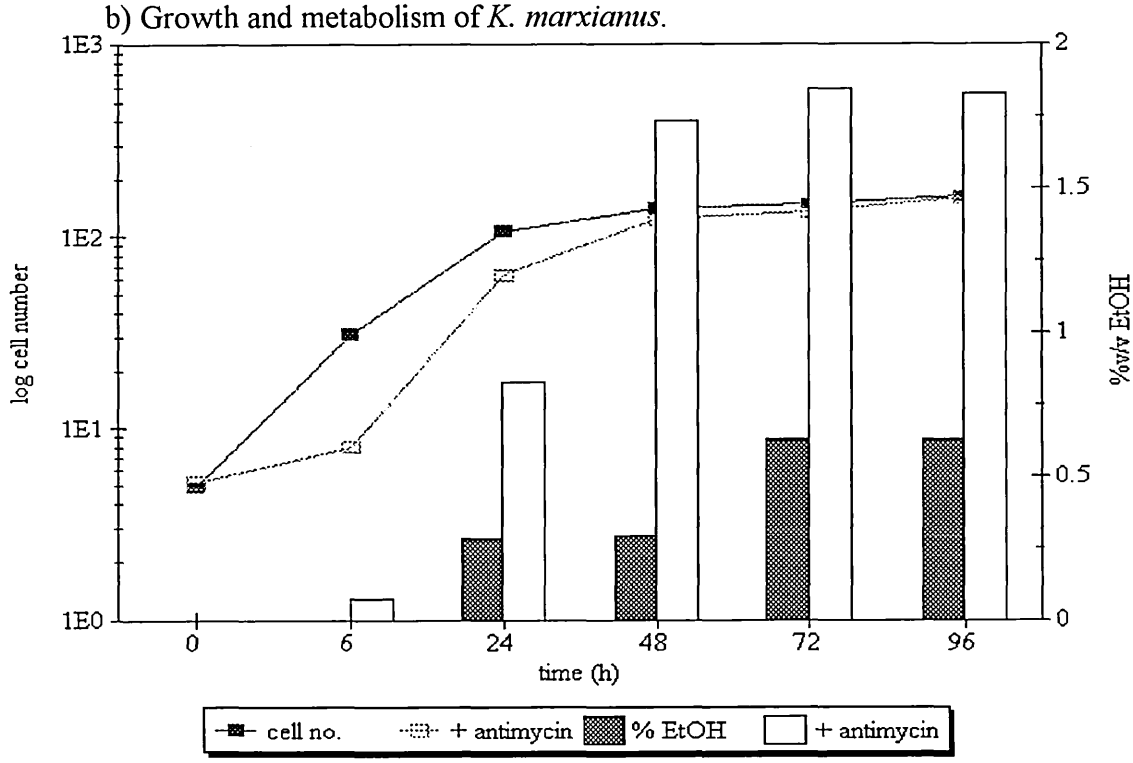
Growth of the non-fermentative yeast *Wickerhamiella domercqiae* (Figure 4.4) was severely inhibited by antimycin A, and this yeast was unable to switch metabolism to counter the effects of the antibiotic. However, the cells did start growing after 24h, suggesting that there exists an alternative respiration existing in this yeast. As there was no noticeable death phase in the culture it would appear that antimycin A stopped its conventional electron transfer route and perhaps, over time, the cells were able to adapt to this by expressing another pathway that allowed them to dispose of electrons and grow, albeit at a slower rate than under normal respiratory conditions.

Figure 4.2 Effect of antimycin A on the growth and metabolism of yeasts growing in batch culture at 50g/l glucose (antimycin A present at 5mg/l)

a) Growth and metabolism of *S. cerevisiae*



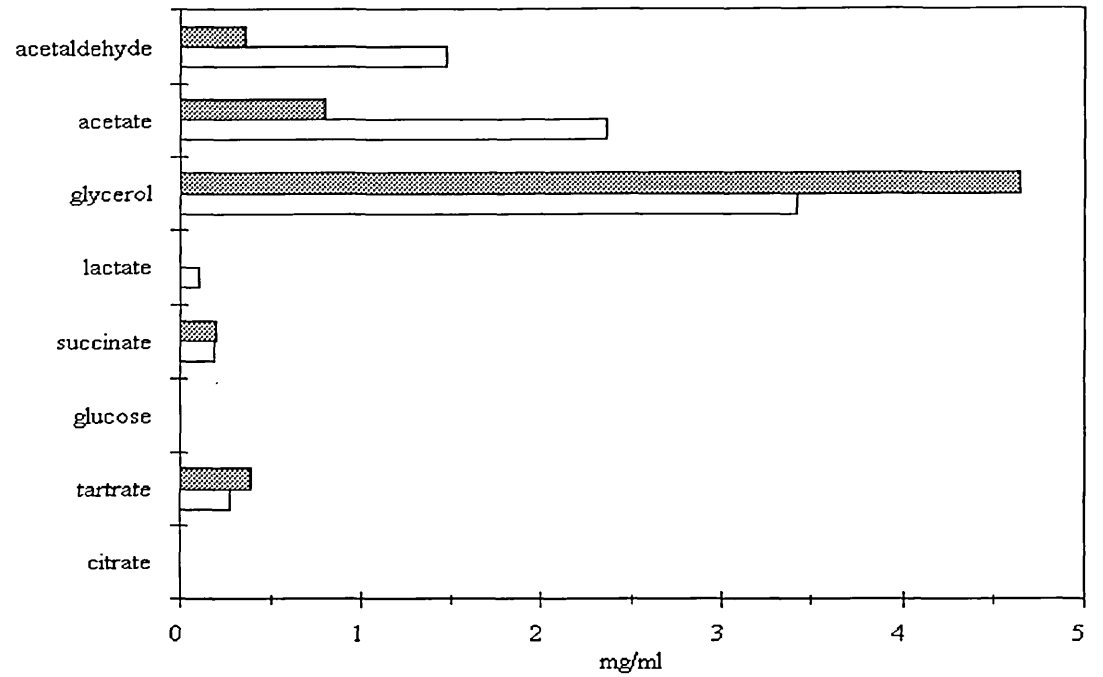
b) Growth and metabolism of *K. marxianus*.



Further evidence for a change in metabolism in those yeasts that were exposed to antimycin A is given in Figure 4.3 where it is apparent that antimycin A influenced the fate of glucose in the affected cultures. Clearly with *S. cerevisiae* there was little effect on production of other metabolites characteristic of respiro-fermentation. Due to the Crabtree effect, there would have been no or little respiratory metabolism present where the conventional electron transport chain for respiration was blocked, through the action of antimycin A. The elevation of glycerol in the presence of antimycin A may be due to the need to replace NAD^+ required in the glycolytic pathway. Decreased extracellular levels of acetate and acetaldehyde may also be due to the cells increased ability to metabolise these products to ethanol rather than utilise them by respiration. In *K. marxianus*, the situation was similar but the changes in metabolism were much more dramatic. This yeast was able to switch to a fermentative metabolism but it also appeared to slow its rate of glucose utilisation. Interestingly, *S. cerevisiae* was able to utilise all the glucose supplied by 96h under both conditions, whereas *K. marxianus* was unable to use all of the glucose under antimycin A-free conditions. In *K. marxianus*, biomass levels were similar at the end of the experiment although there was discrepancy in glucose utilisation which may not be solely due to differences in growth. The switch to completely fermentative metabolism was also reflected in increased glycerol content of the spent medium. This may be explained by internal redox balancing by the cells. *K. marxianus* also showed reduced levels of extracellular acetate but surprisingly decreased levels of succinate. It was not possible to ascertain if the decrease in extracellular succinate was due to utilisation by the yeast, by reduced production through reduced/stalled TCA cycle activity, or even increased reduction to fumarate in an effort to remove electrons through blocked electron transfer (Figure 4.1).

Figure 4.3 Effect of antimycin A on the excretion of metabolites from yeast grown in batch culture at 50g/l glucose (antimycin A present at 5mg/l)

a) Effect on glucose utilisation and metabolites excreted from *S. cerevisiae*



b) Effect on glucose utilisation and metabolites excreted from *K. marxianus*

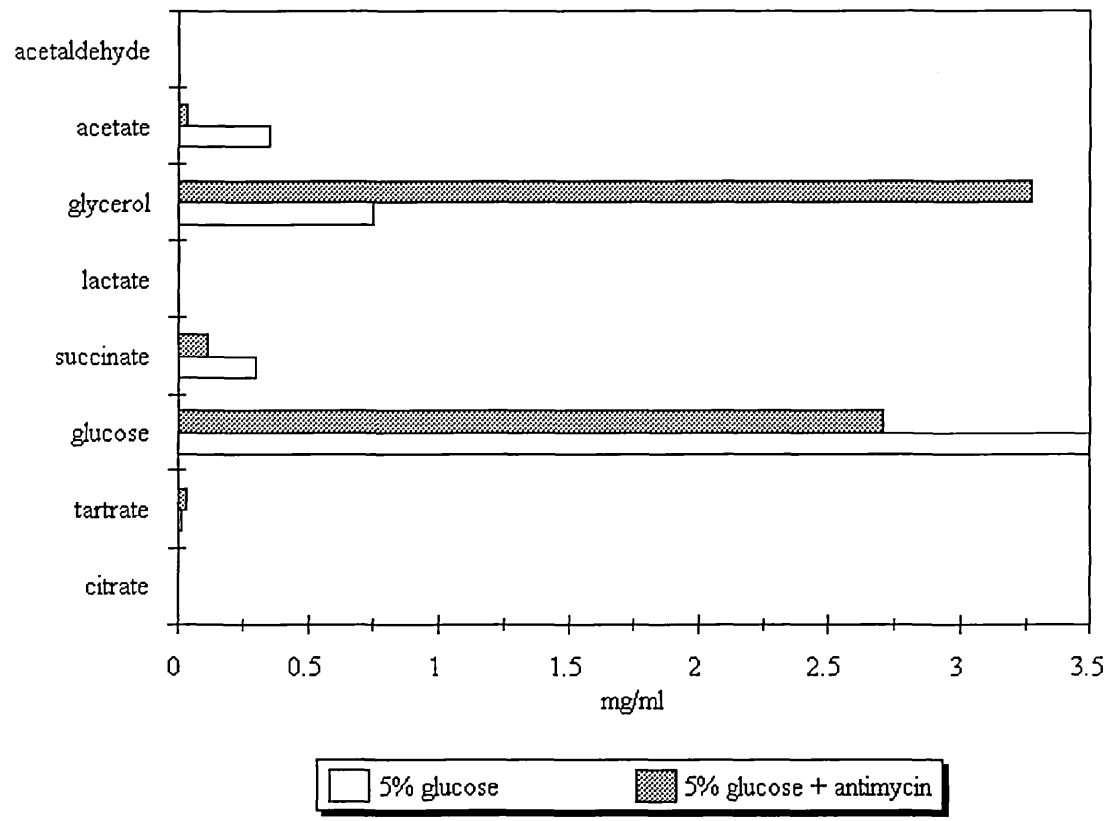
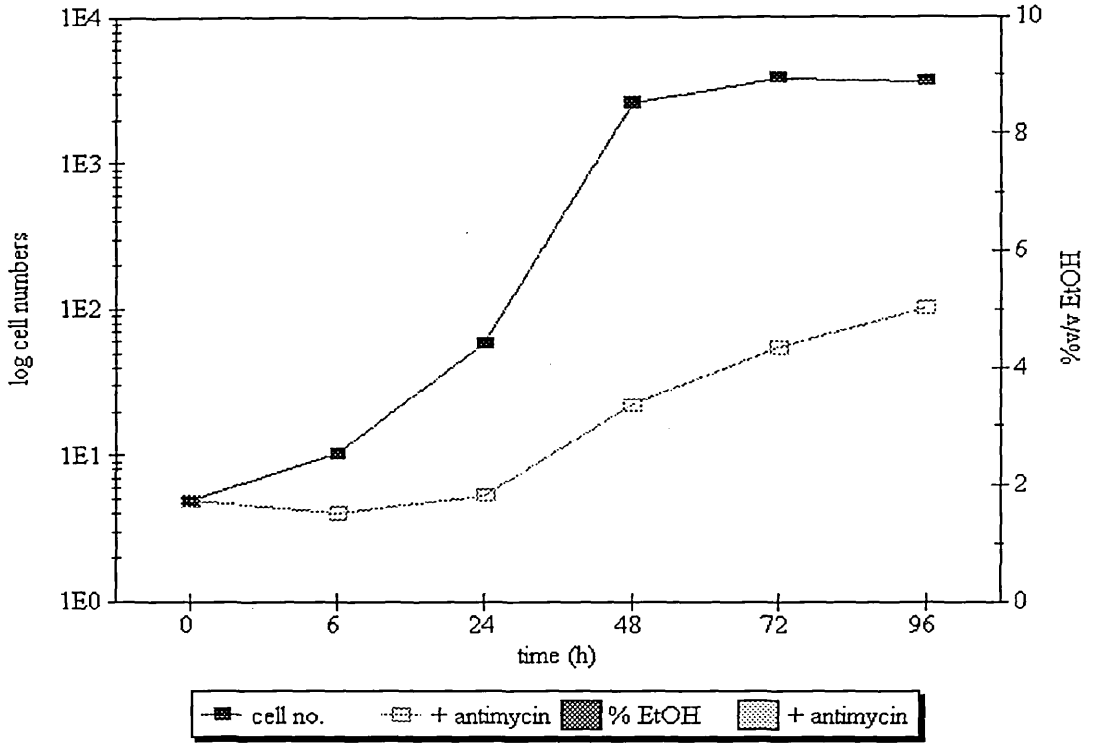
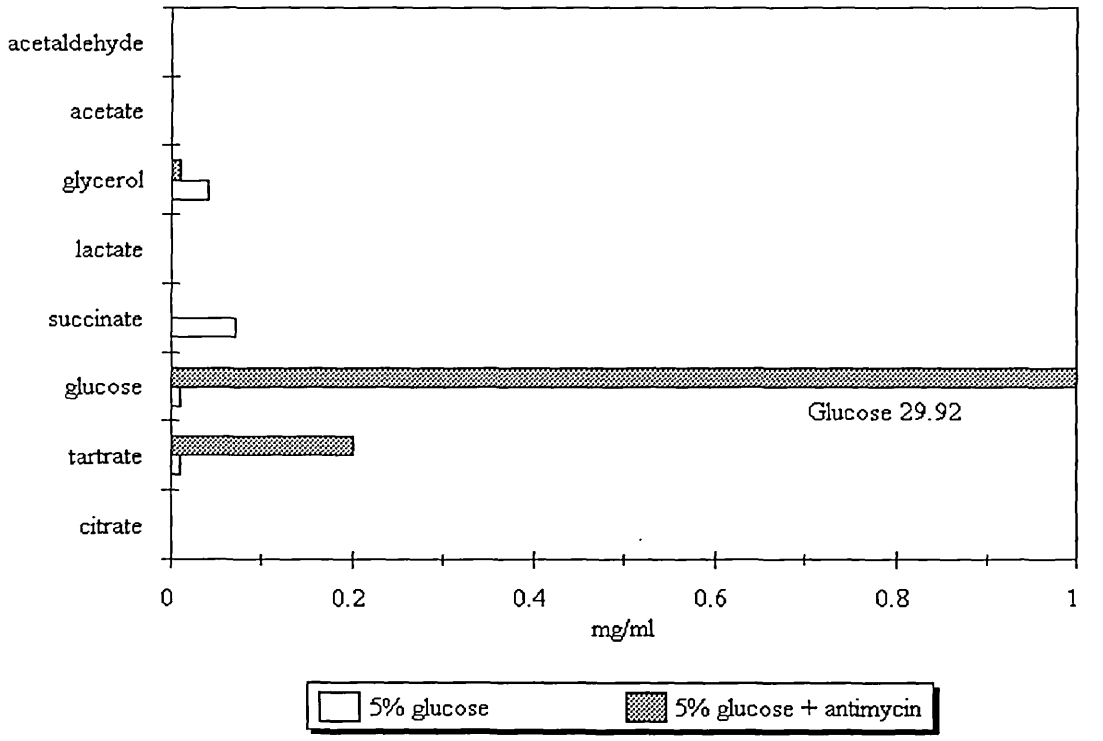


Figure 4.4 Effect of antimycin A on the growth, metabolism and excreted metabolites from *Wickerhamiella domercqiae* grown in batch culture at 50g/l glucose. (antimycin A present at 5mg/l)

a) Effect on growth and metabolism in *W. domercqiae*



b) Effect on glucose utilisation and metabolites excreted from *W. domercqiae*



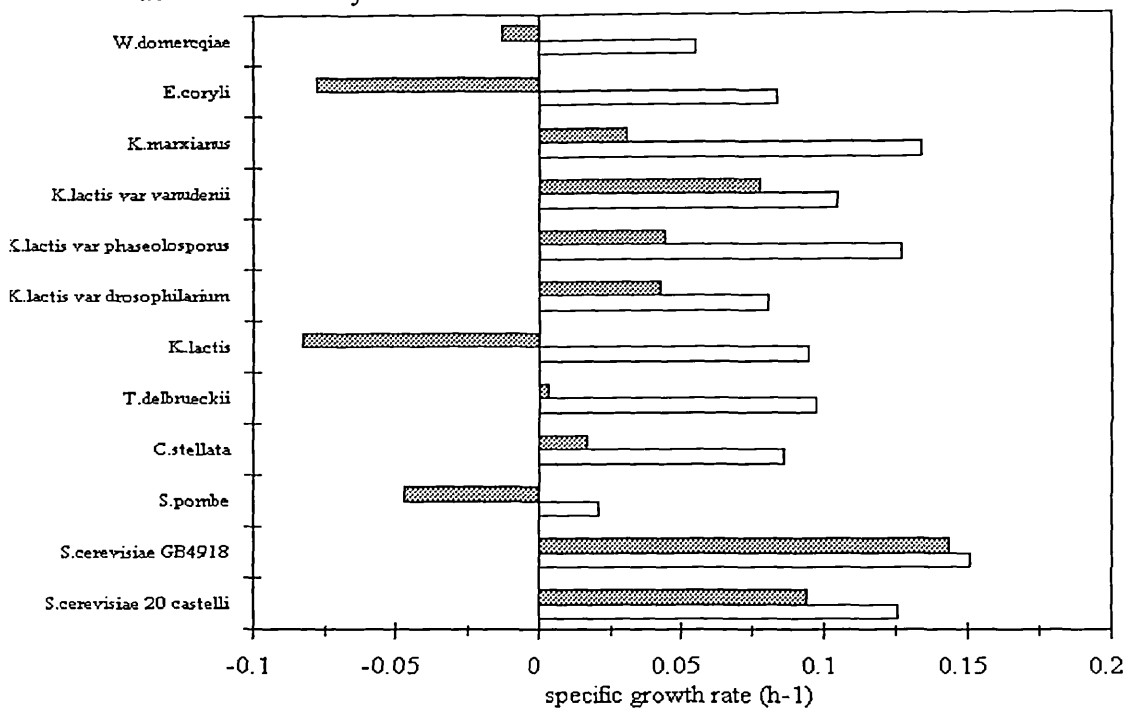
While it was apparent that *W. domercqiae* was able to double cell number several times over the term of the experiment it was clear that the antimycin A had also severely affected its ability to utilise glucose. This can be seen from the high residual glucose remaining at 96h, almost 30g/l, which accounts for 60% of the total glucose the cells were exposed to. In the antimycin A-free culture, cell numbers were less than twice as great and therefore the lack of biomass would not appear to be the reason for reduced glucose uptake.

In Figure 4.5 the effect of antimycin A on the specific growth rates of the all yeast studied over the first 6 and 24h is presented. This data showed that almost all of the yeasts studied were able to grow despite the presence of 5ug/ml of the antibiotic. *Eremothecium coryli* was the only yeast which showed a death rate after 24h, although four yeasts exhibited death over the first 6h on exposure to antimycin A. Even after 96h the growth of antimycin A-treated *E. coryli* was much less than in the control culture. Figure 4.6 shows the effect of antimycin A on the number of doublings undergone by the cultures of yeast during the experiment, which gives a better indication of the effect of antimycin A on growth of the yeast species. Figure 4.6(a) provides a simpler representation of the difference in factorial increase in cell numbers between cells grown in the absence and presence of antimycin A. There was virtually no effect on the proliferation in either of the *S. cerevisiae* species studied or in *K. marxianus.* Surprisingly, there was a great variation of effect on the three *K. lactis* varietal strains that were studied especially when compared in relation to the *K. lactis* type strain. These results do present an argument for the re-evaluation of the taxonomy of these strains on a physiological basis as well as at a genetic level. Another anomaly that arose

was with the yeast *Eremothecium coryli*, which is described as being capable of fermenting glucose (Barnett *et al.* 1990); (Kurtzman and Fell 1998) and exhibiting a Crabtree effect (De Deken 1966). This yeast species failed to produce ethanol at 50g/l glucose and even in the presence of glucose it was unable to continue growing. This is a function which Crabtree positive yeasts should be able to achieve. Oxygen uptake of *E. coryli* (Figure 4.7) was similar to that of the Crabtree positive yeasts, with a significant decrease in uptake occurring when grown in 50g/l glucose. However, most of the yeasts that were capable of fermentation also exhibited some residual oxygen uptake in the presence of antimycin A, suggesting that this may have some role in enabling the cells to continue growing.

Figure 4.5 Effect of antimycin A on the growth rates of different yeast species (antimycin A present at 5mg/l)

a) Effect on growth rate over first 6h exposure compared with growth in the absence of antimycin A.



b) Effect on growth rate after 24h compared with growth in the absence of antimycin A.

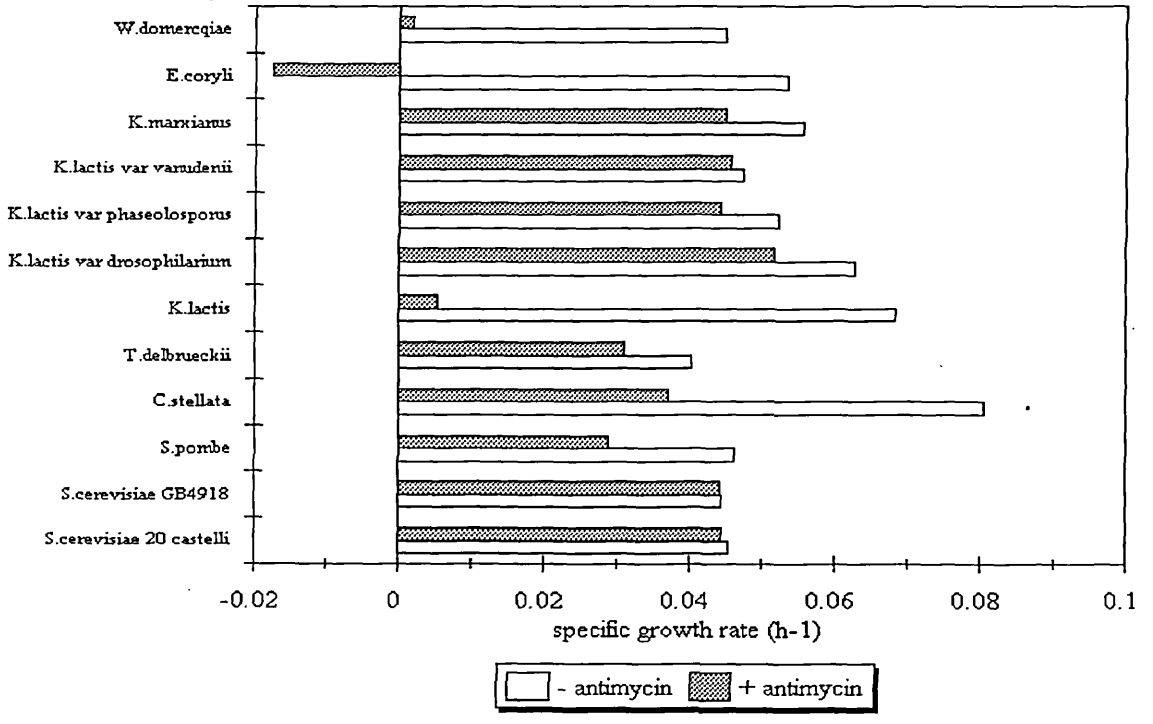
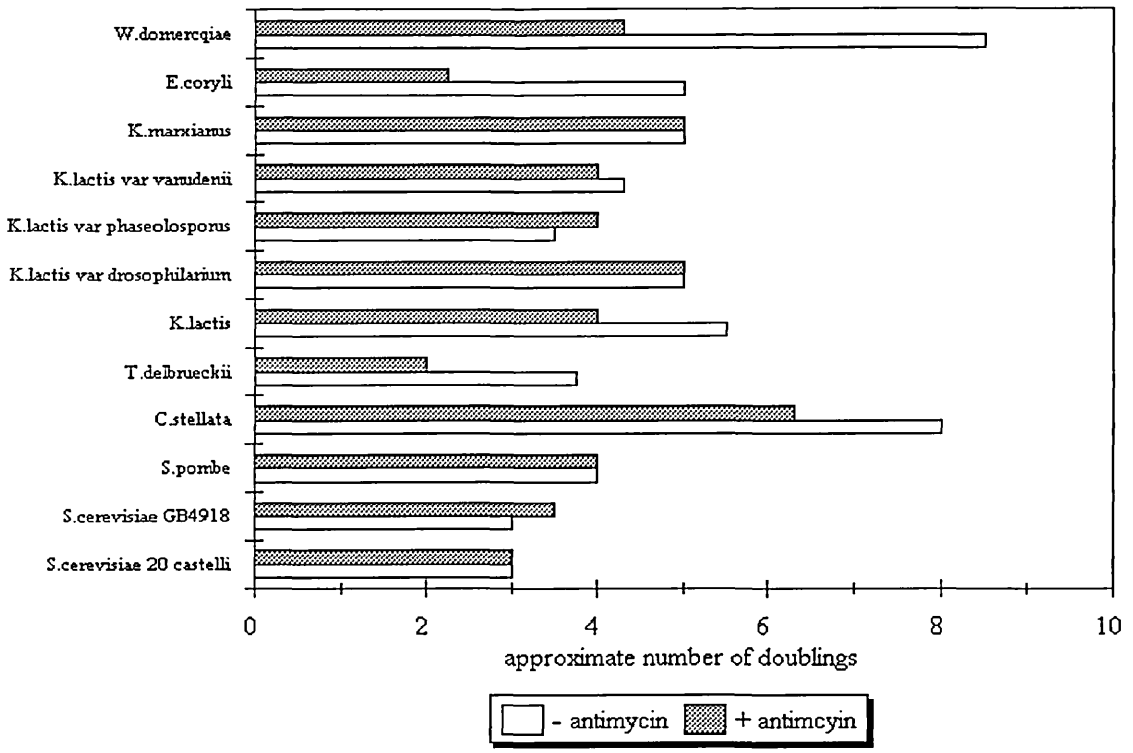
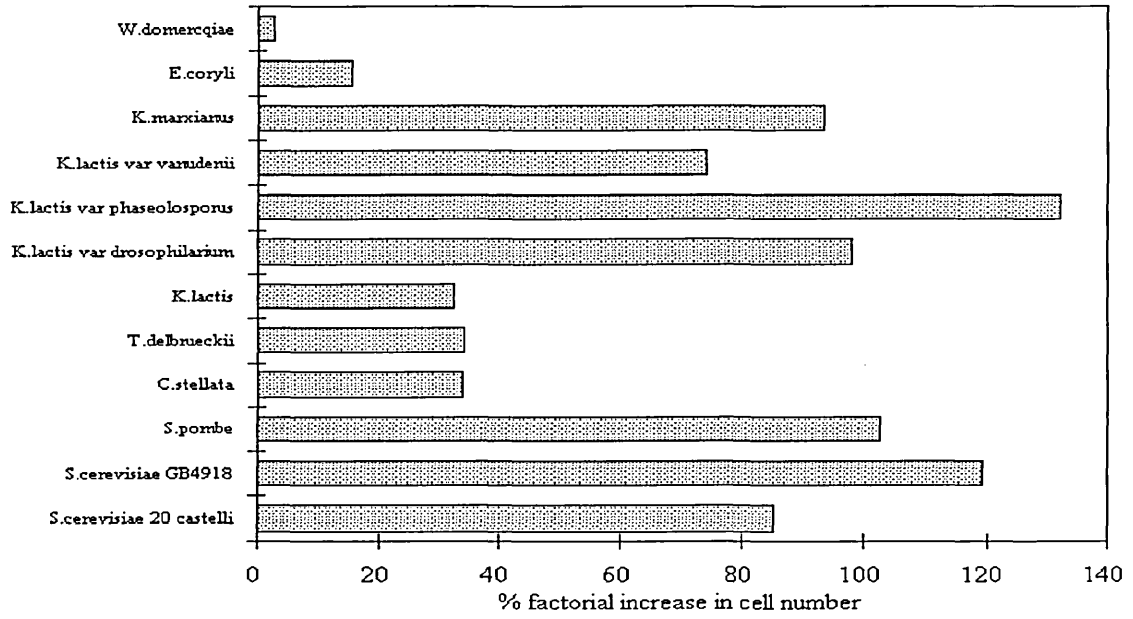


Figure 4.6 Effect of antimycin A on cell number doublings of yeast species grown in batch culture (antimycin A present at 5mg/l)

a) Approximate number of doublings in 50g/l glucose +/- antimycin



b) Doubling in the presence of antimycin with normal growth representing 100% doubling



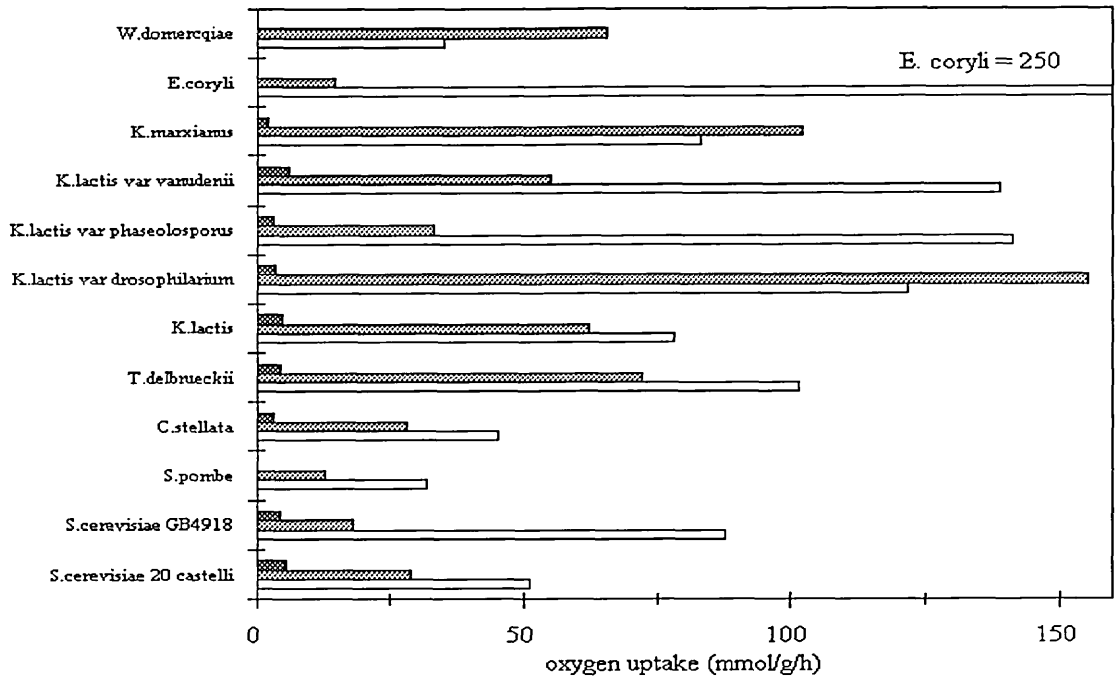
Data in Figure 4.7 relate yeast oxygen uptake in terms of actual uptake per gram of dried yeast biomass per hour, and as a percentage of total oxygen uptake, assuming that cellular uptake rate growing in 1g/l glucose was 100%. Figure 4.7 also showed that *E. coryli* suffered the greatest reduction in oxygen uptake when grown in 50g/l glucose. One reason for the misinterpretation of this yeast as being glucose fermentative (Barnett *et al.* 1990); (Kurtzman and Fell) may be due to the high rate of oxygen uptake it exhibits in 1g/l glucose. This would suggest that even when respiring it could produce a significant amount of CO_2, and in the usual assimilation and fermentation studies used in taxonomy studies this would gather as CO_2 in a Durham tube, leading to the assumption that fermentation had taken place during growth. Measuring alcohol accumulation in the medium by GC or HPLC, provides a more accurate assessment of fermentative metabolism. Research describing *Nematospora coryli* (*E. coryli*) as Crabtree positive as De Deken (1966) used CO_2 evolved against O_2 consumed as a method for determining the activity of aerobic fermentation and therefore the presence of a Crabtree effect. However, ethanol production was not measured. In the batch cultures that produced these results it was recorded that *E. coryli* lacked some other notable fermentation products, glycerol. In the present work, during growth on 50g/l glucose, *E. coryli* did excrete a reasonable amount of acetaldehyde, but lacked ethanol production. When grown in the presence of antimycin A the excretion of acetaldehyde was replaced by a similar level of excretion of acetic acid.

Yeasts determined as non-fermentative and *Schiz. pombe* exhibited a complete lack of oxygen uptake in the presence of antimycin A. All the other yeast strains continued to use oxygen which may suggest that this residual oxygen uptake was due to

the use of molecular oxygen for lipid and sterol biosynthesis. Other researchers (Lagunas 1979) have suggested that the oxygen uptake involved in lipid biosynthesis is an insignificant part of the total uptake since cyanide can reduce oxygen uptake by over 90%. Cyanide is known to inhibit respiratory electron transport chain where the electrons are passed from cytochrome a to oxygen (Figure 4.1). The results presented here agree with those of Lagunas (1979), with antimycin A inhibiting electron transfer further up the transport chain (Figure 4.1). However, in the present work the utilisation of oxygen for lipid biosynthesis was not measured. The lack of oxygen uptake by the yeast *Schiz. pombe*, despite its continued growth and doubling with reduced ethanol output after 96h, suggested that this yeast functioned capably and presumably constructed lipids for membrane synthesis. Alternative forms of respiration have been reported in many yeast species including *Schiz. pombe*, *S. cerevisiae* and *K. lactis* that have continued to exhibit respiration even in the presence of cyanide. This particular form of respiration is known to be sensitive to the inhibitor azide, and is this described as azide-sensitive (AZS). This method of respiration is not fully understood and does not apparently involve electron transfer with the normal respiratory chain at ubiquinone (Alexander and Jeffries 1996).

Figure 4.7 Effect of antimycin A on oxygen uptake of different yeast species grown in batch culture (antimycin A present at 5mg/l)

a) Effect on absolute oxygen uptake.



b) Effect on percentage oxygen uptake assuming uptake at 1g/l to be 100%

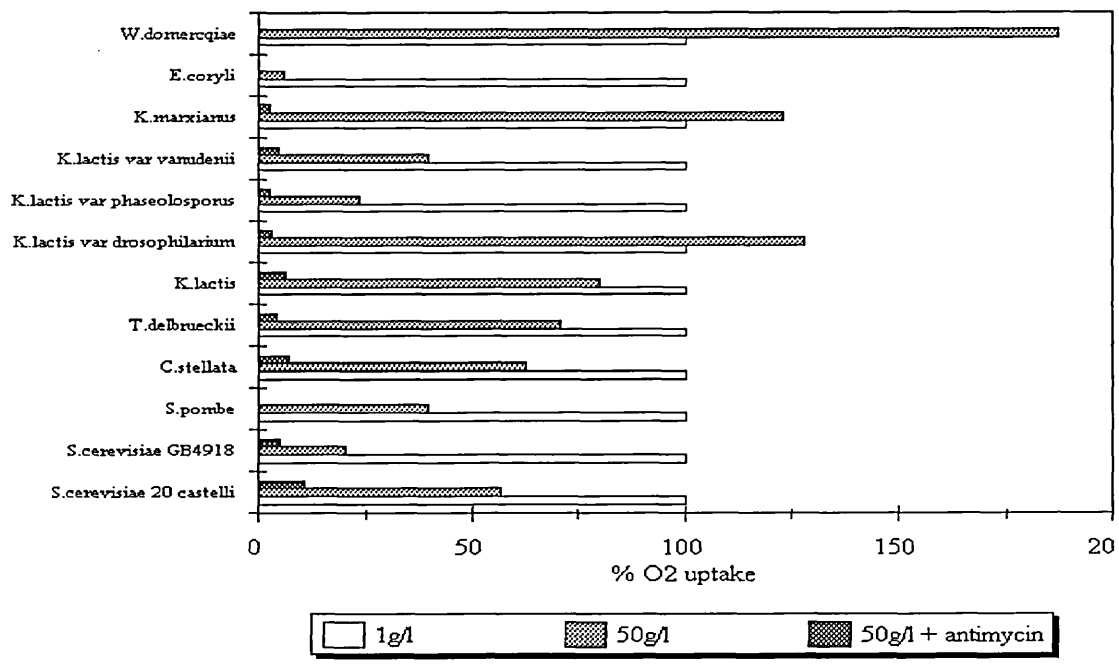
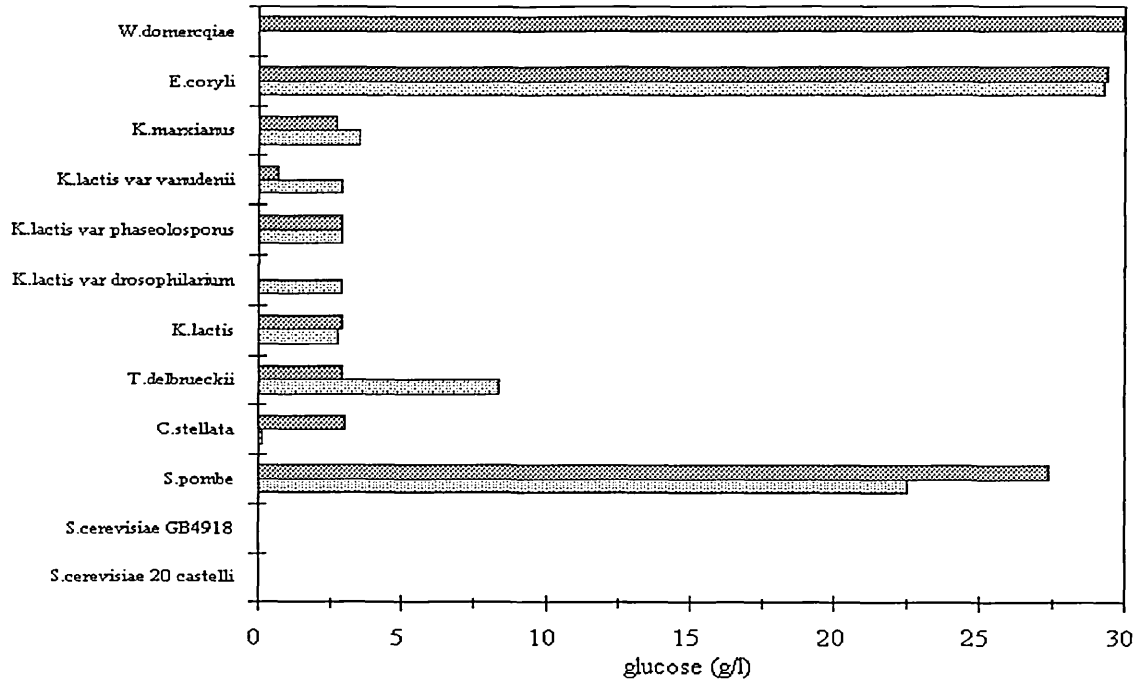
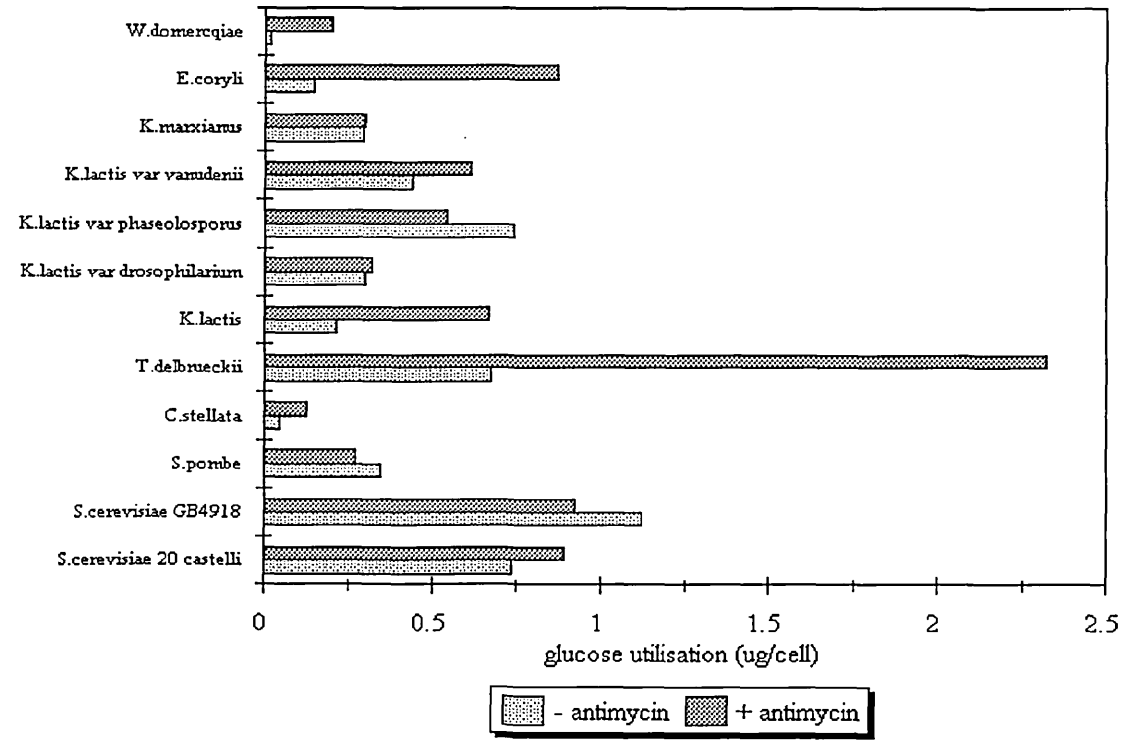


Figure 4.8. Effect of antimycin A on the utilisation of glucose by different yeast species growing in batch culture with 50g/l glucose (antimycin A present at 5mg/l)

a) Effect on residual glucose after 96h growth +/- antimycin



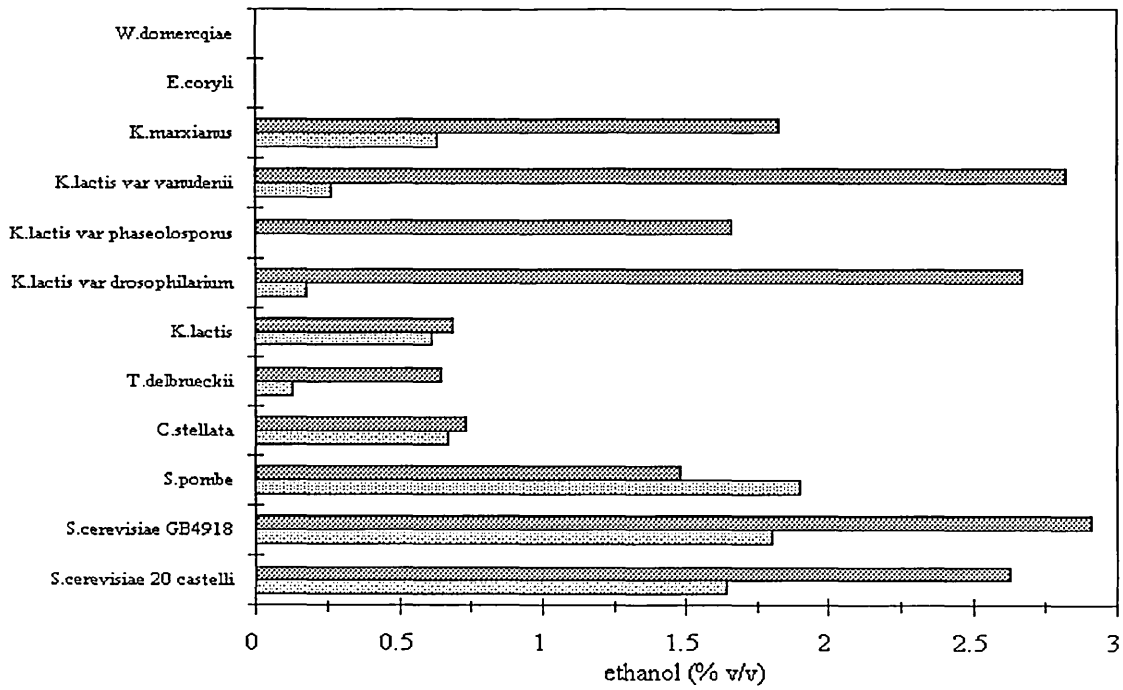
b) Effect on utilisation of the glucose by yeast biomass +/- antimycin



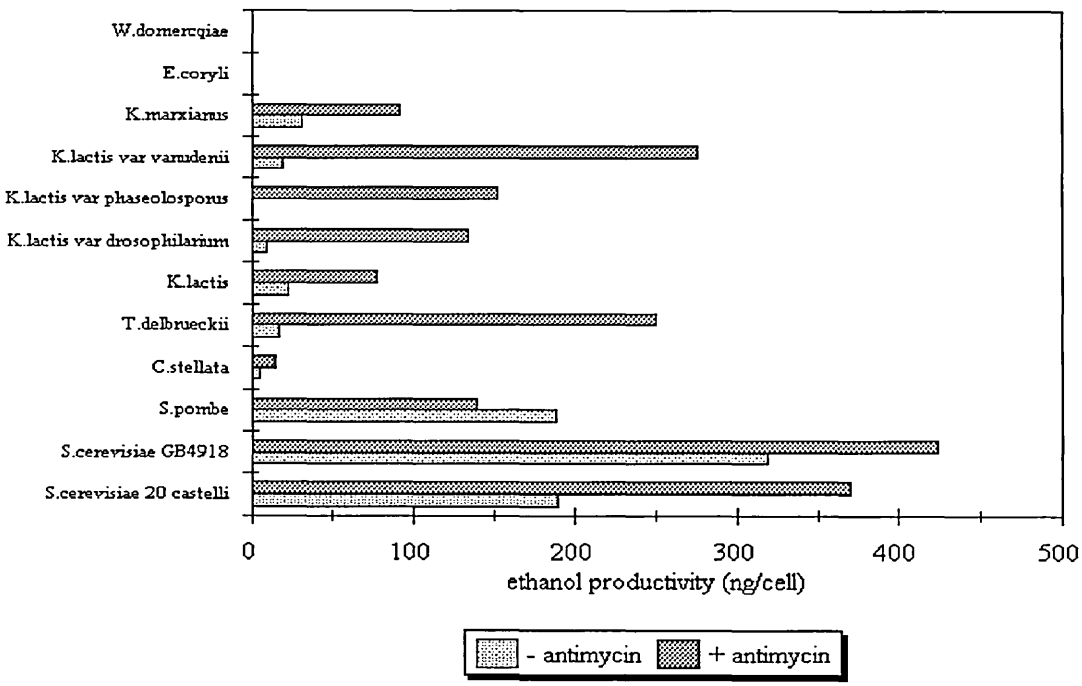
Interesting data on the effect of antimycin A on utilisation of glucose by different yeasts is shown in Figure 4.8. The first assumption that was established was that in subjecting Crabtree positive yeasts to growth in the presence of antimycin A there should be no great change in the utilisation of the glucose. The effect of the antibiotic would have been to close down the flow of electrons through the respiratory chain, a process that Crabtree positive cells would carry out to a limited extent. The results in Figure 4.8 agree with this hypothesis, and in the case of a wine strain of *S. cerevisiae* (20 castelli), there was a slight increase in the use of glucose per cell, due to the slight reduction in cell numbers (results not presented). What this does mean is that increase in cellular glucose utilisation per cell must be due to increased metabolism when the cells are fewer in number. The existence of alternative respiration was postulated as a possible reason for the increased fermentative performance of a commercial brewing yeast (Lodolo *et al.* 1999) although in this case the researchers concluded that increased fermentation was due to increased yeast growth, which does not tally with results presented here. Lodolo *et al* (1999) did not actually determine yeast growth and mainly inferred from results showing reduction in wort gravity, maltose utilisation and slight recovery of cells inhibited in the presence of azide. In the present work, increases in glucose utilisation per cell in the presence of antimycin A were observed in *Torulaspora delbrueckii*, *K. lactis*, *E. coryli*, and *W. domercqiae*. These results concur with data presented in Figure 4.9 for *T. delbrueckii* which exhibited a greater ethanol productivity per cell. They did not concur, however, for the other species that exhibited this increase in glucose usage per cell. It is also noteworthy that of the three yeasts that did not respond with an increase in ethanol productivity, only two are classed as non-fermentative (i.e. *E. coryli* and *W. domercqiae*).

Figure 4.9 Effect of antimycin A on the production of ethanol by different yeast species grown in batch culture with 50g/l glucose (antimycin A present at 5mg/l)

a) Effect on ethanol produced



b) Effect on cell ethanol productivity



Although all three yeasts (*K. lactis, E. coryli* and *W. domercqiae*) suffered a death phase in the first 6h in the presence of antimycin A (Figure 4.5), only *E. coryli* continued to die up until 24h. All four yeasts (*K. lactis, T. delbrueckii, E. coryli* and *W. domercqiae*) that have displayed increased glucose utilisation per cell also suffered greatly reduced cell division compared with cells grown in the absence of antimycin A. In fact, the performance of these yeasts may be due to an adaptive phase that the cells have had to go thorough which stalls their growth.

From these results it became clear that expression of the Crabtree effect in yeasts grown in chemostat culture would be best carried out on yeast species which behaved in a predictable fashion in shake flask culture. A prime Crabtree positive candidate would be one of the strains of *S. cerevisiae,* and since baker's strain GB4918 was used throughout this thesis, this was the species of choice. From the data presented previously, it was clear that this particular strain exhibited a strong Crabtree effect, and also possessed a greater fermentative capacity than the other strain of *S. cerevisiae*. Of the Crabtree negative species, only *C. stellata* and *K. marxianus* showed the consistency expected of them in these batch cultures. Although, *K. marxianus* showed the greatest increase in ethanol production in the presence of antimycin A, suggesting that the growth of *C. utilis* was inhibited to a greater extent by the presence of the antimycin A. This lead to the use of *K. marxianus* as the Crabtree negative yeast to be used in the chemostat studies. *K. marxianus* is also a species with several industrial applications (e.g. production of ethanol from cheese whey fermentations).

4.3.2. Growth of *S. cerevisiae* and *K. marxianus* in glucose-limited chemostats subjected to a pulse of glucose.

The main purpose of this section was to identify any physiological characteristics that may differ in Crabtree positive and Crabtree negative yeasts. Experiments were carried out in chemostat cultures and analytical procedures were similar to those carried out in Chapters 3,4, and 5. The first characteristic of interest was the physical change in cell volume that occurred as glucose levels were rapidly increased. Figure 4.10 shows this most effectively, with *S. cerevisiae* exhibiting a massive increase in cell volume from about 75femtolitres (fl) to over 220fl after 24h when the glucose concentration was at its highest. This represents a 3-fold increase in mean cell volume of the cultures. For *K. marxianus*, there was, similarly, an increase in cell volume but in this case from 20fl to 32fl at about 6h falling back to 28fl at the maximal concentration of glucose. This represents a 1.5-fold increase in cell volume, not as large as observed in *S. cerevisiae*. The mean cell volume in *S. cerevisiae* was about 3-fold larger than that of *K. marxianus*, under glucose-limiting conditions, and once this limitation was removed the difference in mean cell volumes between the species increased to 7-fold. The difference in cell volumes in the two yeasts continued even when the feed medium glucose level returned to 1g/l. Cells in the culture of *S. cerevisiae* reduced their volume very quickly, whereas the change in *K. marxianus* volumes was much more gradual.

Crabtree positive and negative yeasts also differ in the manner in which they accumulate biomass in response to altered glucose availability. In *S. cerevisiae*, cells increase biomass concentration at the same rate they increased cell volume, suggesting

that this may be mainly due to an increase in cellular macromolecular biosynthesis accompanying the increase in size. *K. marxianus* cultures did not exhibit this relationship suggesting that the rapid increase in cell volume was not reflected in increased synthesis of yeast macromolecules. It is noteworthy that the increase in yeast biomass in both cultures was of the same magnitude (about 5-fold), with *S. cerevisiae* gaining biomass at a quicker rate, in the early stages, compared to *K. marxianus*.

At this point the idea was formed that the increase in cell volume may be related to the Crabtree effect. This would be evident in the behaviour of the cells with regard to the fate of the extra glucose with which they were supplied during pulsing. This was further investigated and results showing the fate of glucose in these two yeasts is presented in Figures 4.11 and 4.12. These data provide another interesting difference between Crabtree positive *S. cerevisiae* and Crabtree negative *K. marxianus*. For example, it is clear that *S. cerevisiae* responded to the increased availability of glucose by fermenting it, and as the level of glucose continued to increase so did the amount of accumulated ethanol. Lack of a significant biomass yield resulted from this initiation of ethanol production, as was expected. *K. marxianus* behaved differently, with biomass accumulation being favoured at the expense of ethanol formation. Some production of ethanol was recorded in *K. marxianus* cultures 12h following a glucose pulse but this was unlikely to be due to oxygen limitation.

Figure 4.10 Effect of a glucose pulse on the mean cell volume and biomass accumulation of yeast grown in chemostat culture at $0.15h^{-1}$.

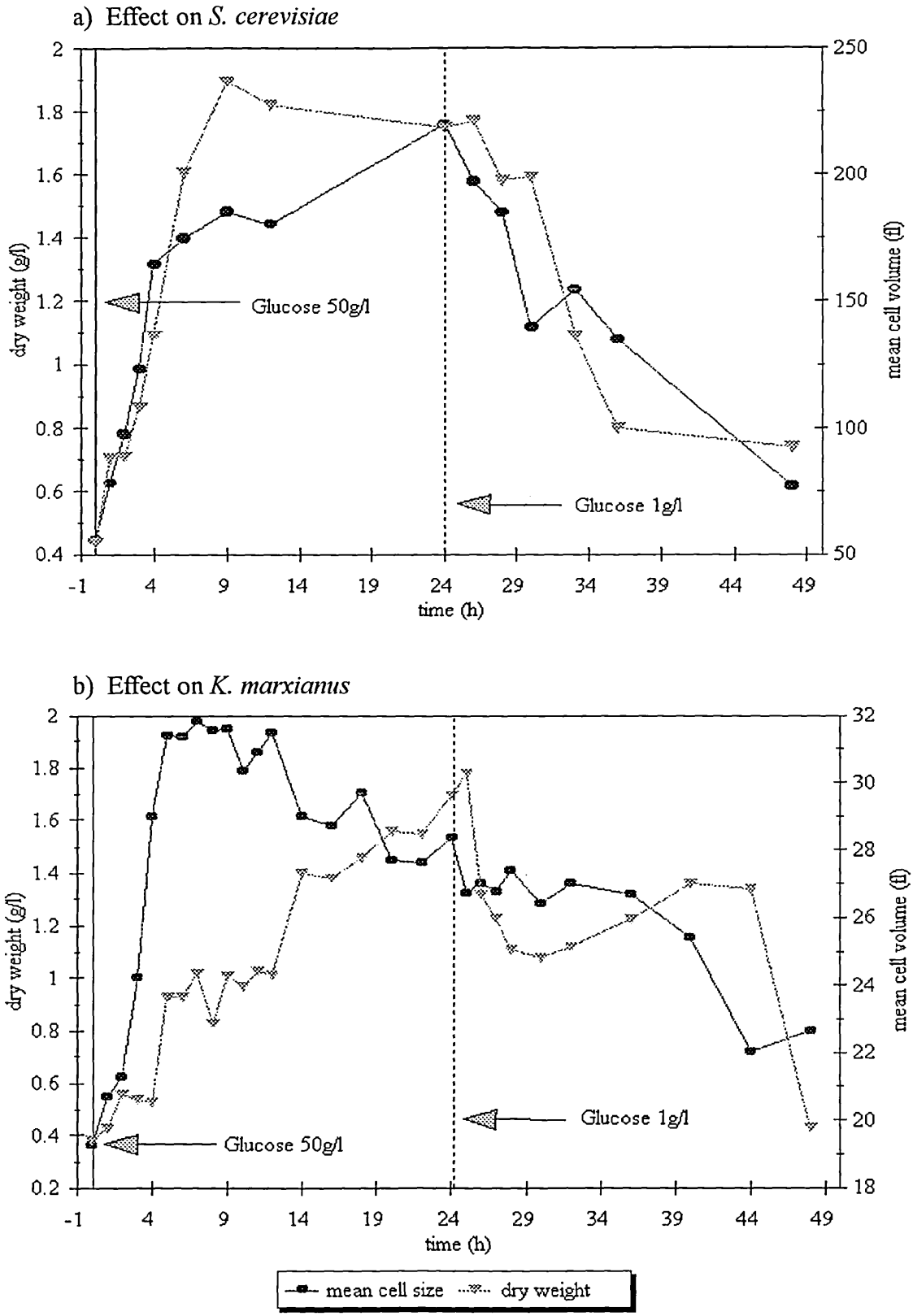
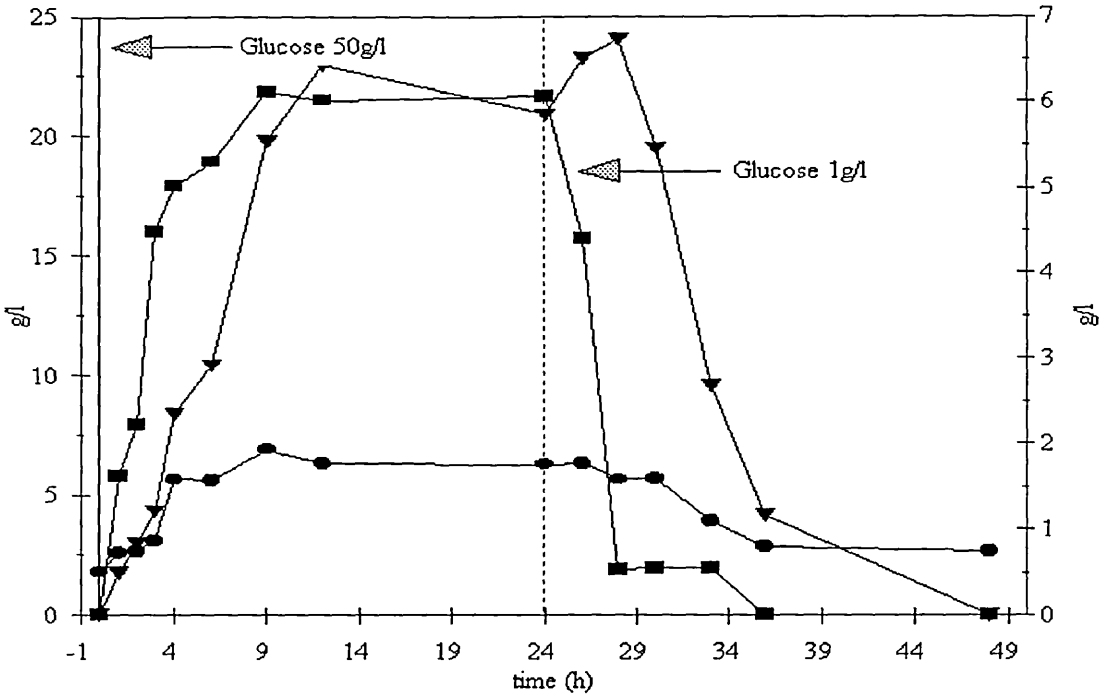
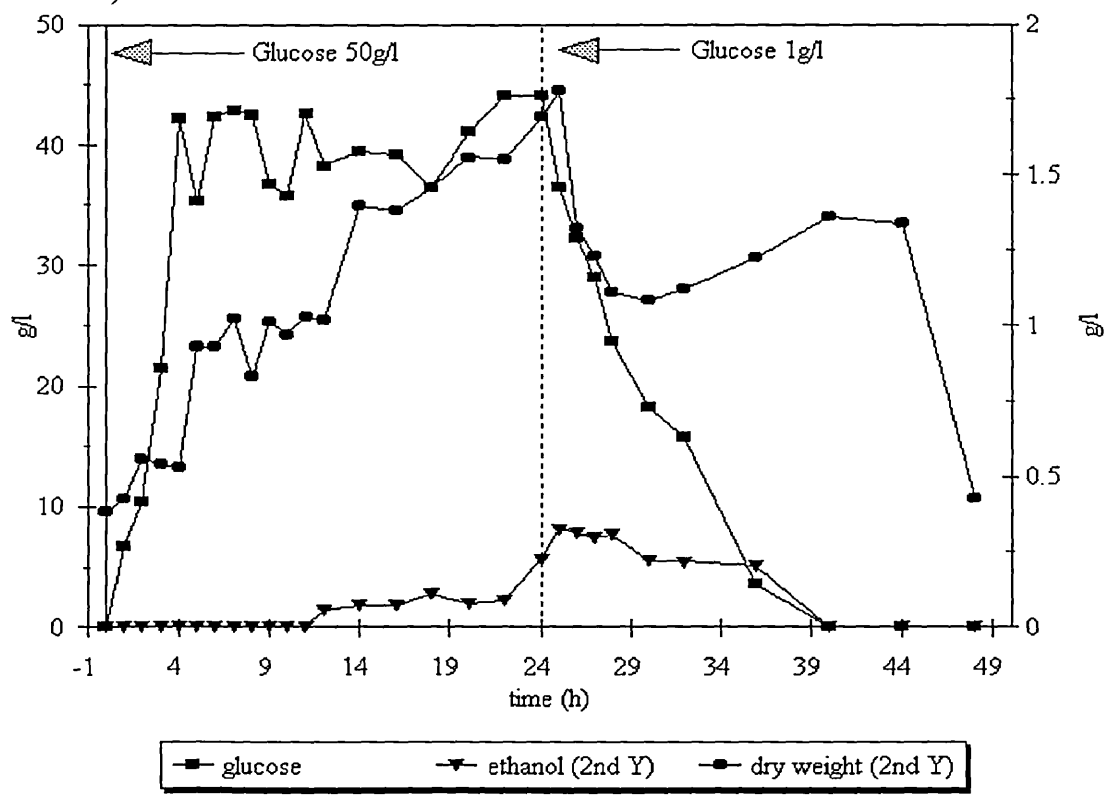


Figure 4.11 Effect of glucose pulse on the fate of glucose in chemostat cultures of yeast at $0.15h^{-1}$.

a) Effect on *S. cerevisiae*

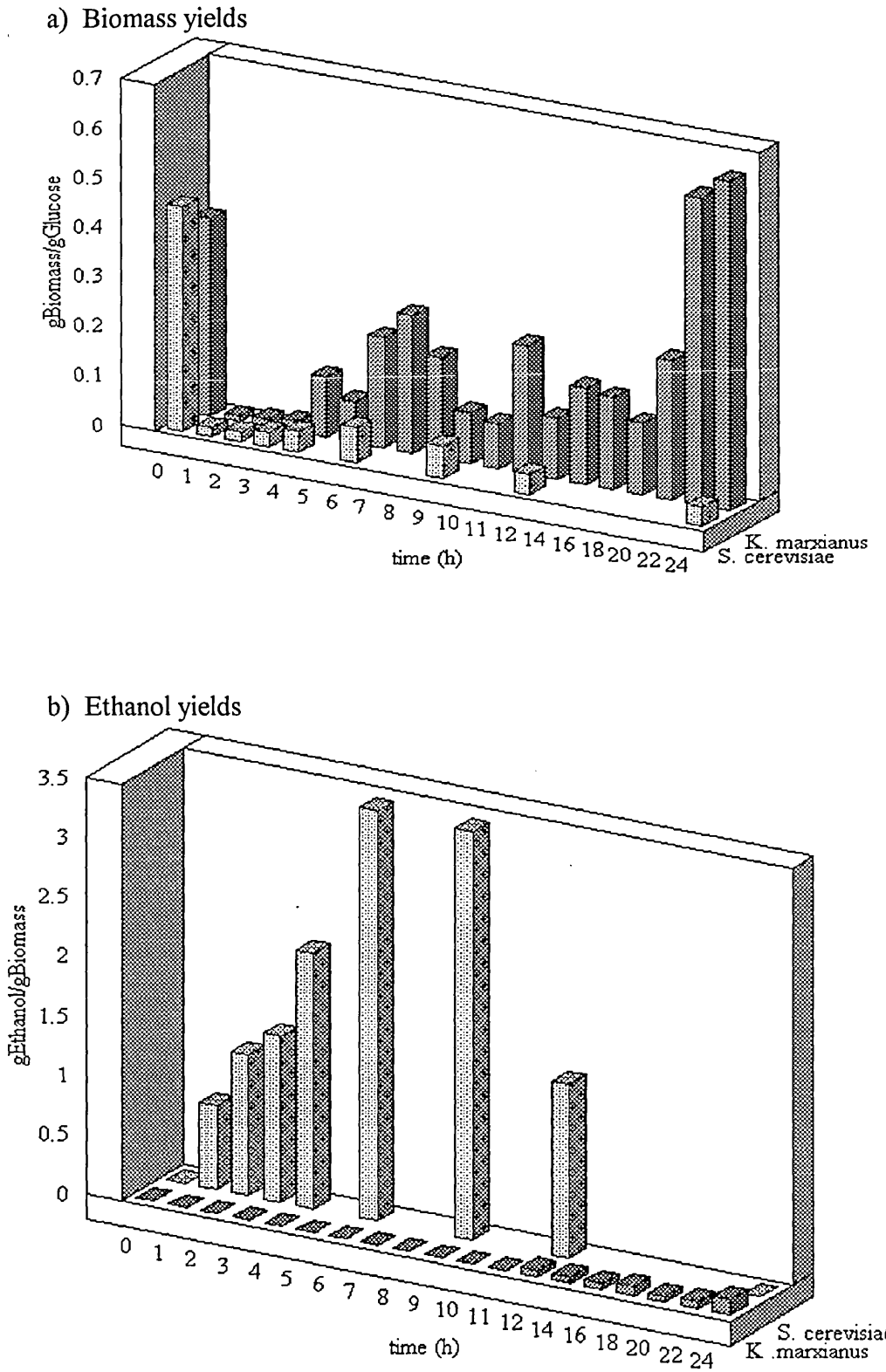


b) Effect on *K. marxianus*



It was also evident from this data that these yeasts have different capacities for metabolising available glucose. Both cultures were supplied with 50g/l glucose and although it was possible to estimate what the cells would do with this excess it was not expected that both species would be unable to utilise all the glucose they were supplied with. In this regard, significant discrepancy was observed between the two yeast strains. *S. cerevisiae* was able to utilise more than half of the glucose with which they were supplied, as measured by the residual glucose in the spent medium in the first few hours following the pulse. *K. marxianus* was able to use only about 10% of the glucose supplied to it within 4h where 42g/l glucose passed through the chemostat unused. At the same sample point *S. cerevisiae* allowed only 18g/l to pass through the system un-utilised. It is possible that the increase in cell volume had an effect on the glucose uptake of the two yeasts. It would appear that the increased glucose concentration is more effectively used by *S. cerevisiae*, with both biomass and ethanol production contributing to removal of more than half of the available glucose. This would suggest that the behaviour of a Crabtree positive yeast is one that confers upon it an advantage that other organisms are incapable. The rapid increase in cell size that is seen here is consistent with results in Chapter 3 where it was shown that cell volume in *S. cerevisiae* appeared to be linked with the way in which the cell metabolised available glucose. In contrast, *K. marxianus* cells appeared to have re-acted to a lesser extent to the increased available glucose, with marginal changes in cell size possibly due to osmotic-stress responses.

Figure 4.12 Biomass and ethanol yields during glucose pulse into chemostat cultures of *S. cerevisiae* and *K. marxianus* grown at $0.15h^{-1}$.



In returning to normal glucose levels *S. cerevisiae* appeared to react as expected with ethanol levels decreasing as glucose became depleted, both through metabolic utilisation and by the physical process of washout from the chemostat. Biomass levels also slowly drop away, although by the end of the experiment there is not a return to a pre-pulse biomass level. It is also apparent that at the end of the glucose pulse *S. cerevisiae* rapidly depleted glucose levels, whereas in the cultures of *K. marxianus* the rate of glucose depletion was much more gradual. There was also a retention of biomass levels above 1g/l in *K. marxianus* cultures, despite the reduction in available glucose. This remained the case until residual glucose level reached zero and the cells were incapable of maintaining such a yield of biomass from the level of glucose supplied.

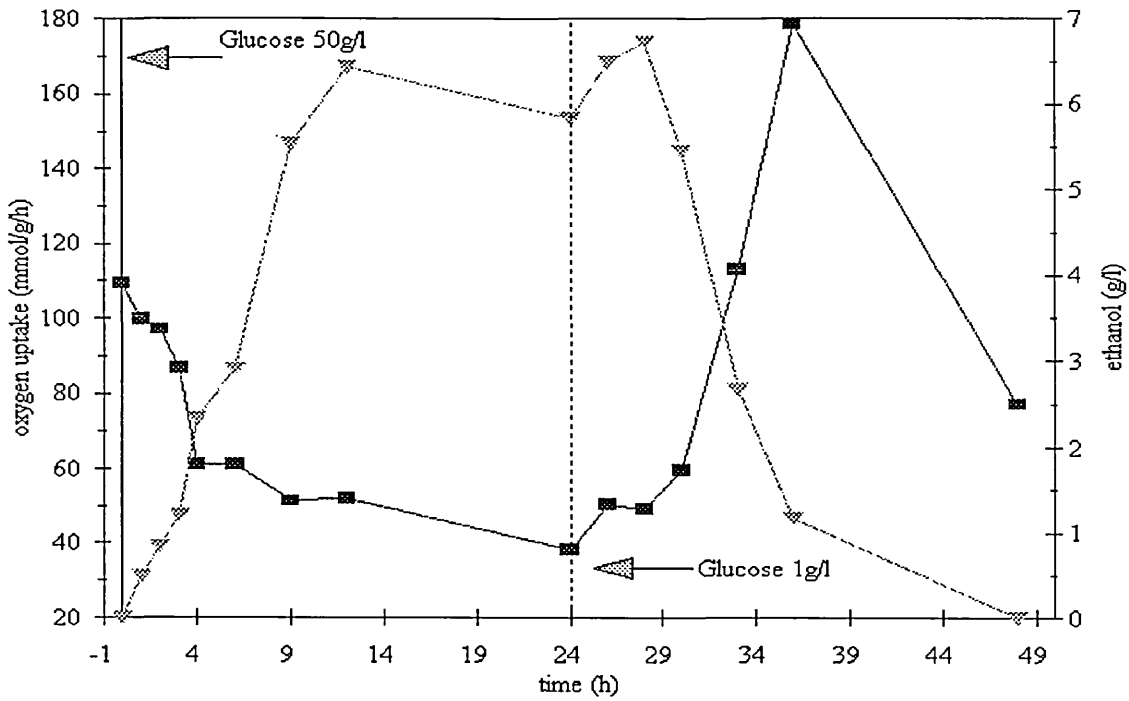
This difference in glucose utilisation between *S. cerevisiae* and *K. marxianus* is more clearly shown in Figure 4.12 where the yield of biomass and ethanol for each yeast can be calculated from the residual glucose passing out of the chemostat. This indicated that both yeasts were behaving as expected of a Crabtree positive and Crabtree negative yeast. In Figure 4.12 a) it was clear that *K. marxianus* utilised most of the glucose for the production of biomass with yields approaching 0.65g biomass/g glucose(gB/gG). Prior to the glucose pulse, both yeasts started off with biomass yields about 0.45gB/gG, which represents good conversion, but *S. cerevisiae* quickly reduced to a biomass yield below 0.1gB/gG. This is consistent with a fermentative mode of metabolism. This was further illustrated in Figure 6.12 b) where the roles were reversed with *S. cerevisiae* providing ethanol yields upwards of 4g ethanol/g biomass (gE/gB). Even in the later stages of a 50g/l glucose pulse *K. marxianus* was incapable of producing significant amounts of ethanol from the biomass present. This suggested that the production of

ethanol in this culture may not be due to oxygen limitation but perhaps due to an overflow in its respiratory pathway, somewhat akin to that suggested by authors studying the respiratory capacity of *S. cerevisiae* (Van Hoek *et al.* 1998a).

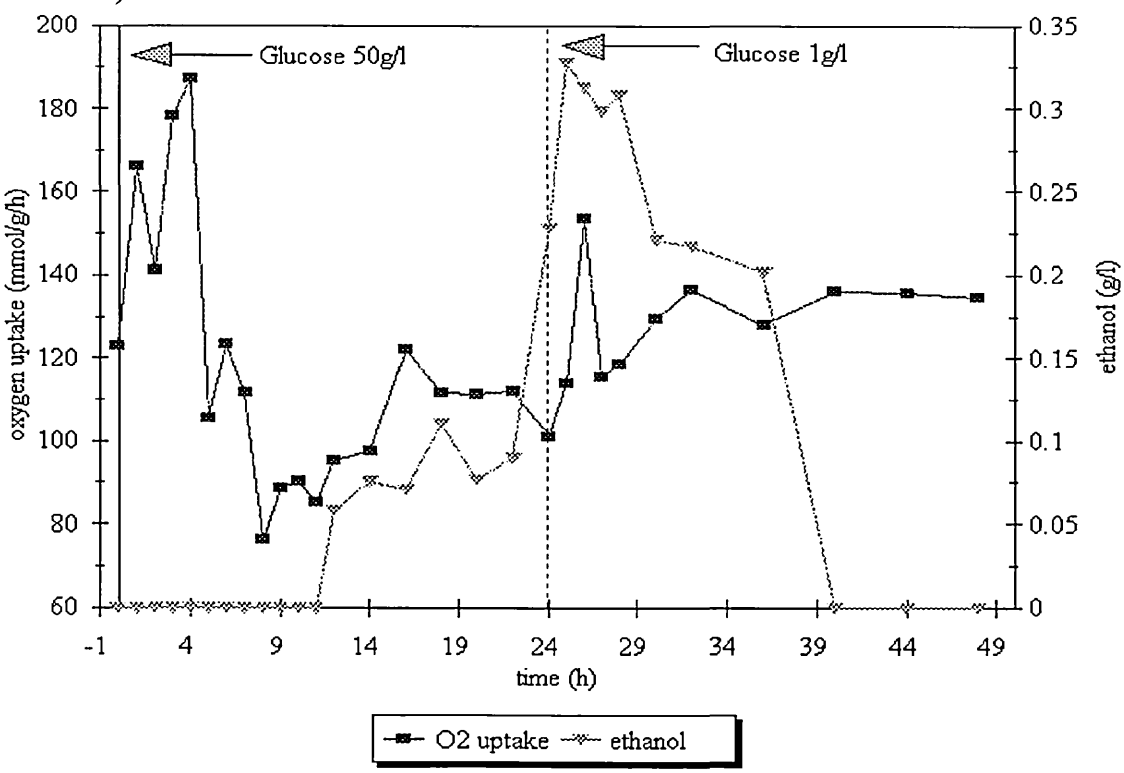
Oxygen uptake of the yeasts during glucose pulsing in a chemostat was also recorded and provided additional information about the Crabtree phenomeon. Data presented in Figure 4.13 show that both yeasts exhibited a decrease in overall oxygen uptake following a glucose pulse, but neither yeast completely abolished oxygen uptake. Surprisingly, once the rate of oxygen uptake had reached its lowest point, *K. marxianus* exhibited a small amount of ethanol production. It is clear that respiratory metabolism ensues as a small amount of ethanol is produced. With *S. cerevisiae*, oxygen uptake only started to increase when the feed contained 1g/l glucose and the subsequent levels of glucose in the chemostat were being rapidly removed. It was interesting, however, that *S. cerevisiae* appeared to raise its oxygen uptake well beyond the level of oxygen uptake that was recorded before the pulse of glucose was begun. This 'over-compensation' was reduced as residual ethanol levels decreased. This occurred at 36h, which was also the time at which residual glucose returned to zero, suggesting that this increased oxygen uptake may be due to the dual utilisation of both glucose and ethanol as the cells became de-repressed from the effects of glucose on respiratory enzymes and on the enzymes of the glyoxylate cycle that are required for growth on ethanol (Zimmermann and Entian, 1998).

Figure 4.13 Effect of glucose pulse on oxygen uptakes of yeasts grown in chemostat cultures at $0.15h^{-1}$.

a) Effect on *S. cerevisiae*



b) Effect on *K. marxianus*



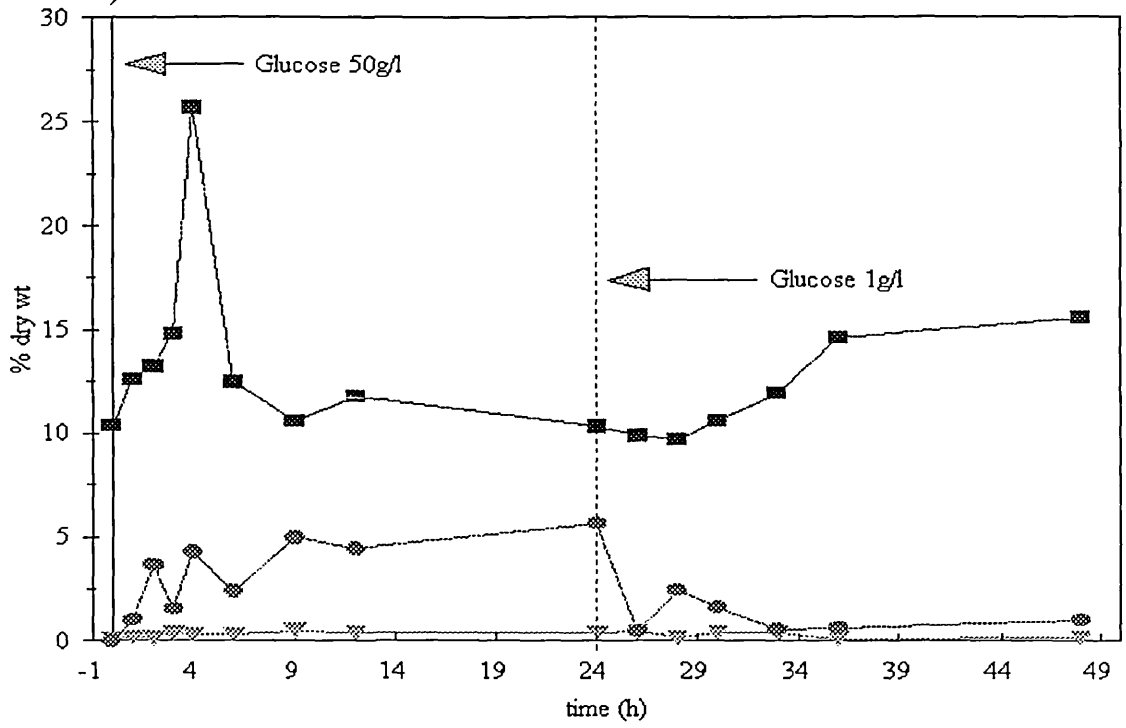
Once the residual glucose reached zero there remained only very low levels of ethanol and so oxygen uptake may be reduced to compensate for the reduced levels of substrate. Perhaps the level of ethanol in the chemostat was reduced to zero before 48h, (although no sample was taken between 36 and 48h) and this may be responsible for making the cells appear as if they have continued to ferment, and therefore prolonging the time taken for the residual ethanol level to reach zero.

The effect of raising glucose concentration on intracellular compounds was most evident in cultures of *S. cerevisiae*, where protein levels were seen to rise over the first 4h before returning to pre-pulse levels by 9h. After the switch back to 1g/l glucose in the feed medium, the level of cellular protein increased slightly. This increase was also seen in the *K. marxianus* cultures, despite protein levels remaining constant after the initial pulse of glucose (Figure 4.14).

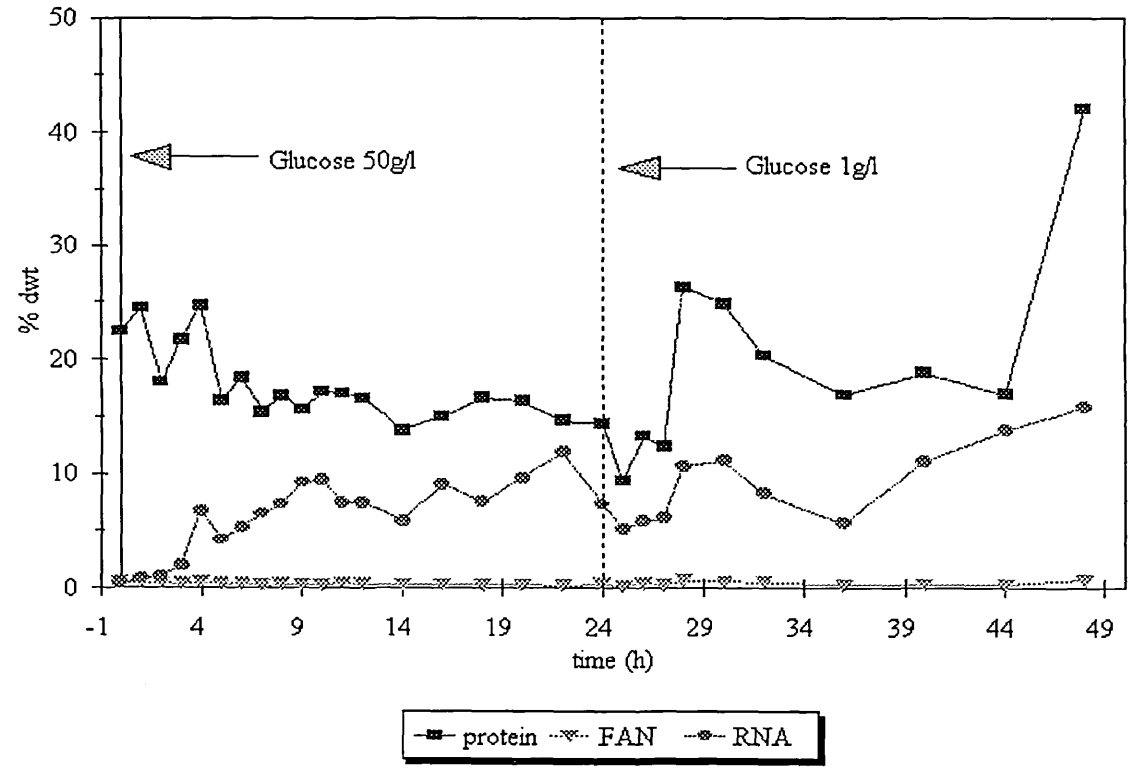
Analysis of cellular RNA also revealed differences between the two yeast species studied. *S. cerevisiae* appeared to increase RNA levels after the increase in glucose to 50g/l. This may be consistent with the increase seen in protein levels, although it is possible that the increase in RNA levels could also be due to a slower rate of RNA biosynthesis or reduced turnover in the cells as glucose levels increased. However, the rapid switch in metabolism exhibited by *S. cerevisiae* (i.e. from respiratory to respirofermentative to fermentative) would almost certainly require increased production of glycolytic enzymes. This fact is alluded to in data presented earlier (Figure 4.11) where *S. cerevisiae* was able to utilise the available glucose much more readily than *K. marxianus*.

Figure 4.14 Effect of a glucose pulse on the intracellular levels of protein, FAN and RNA on yeast growing in chemostat cultures at $0.15h^{-1}$.

a) Effect on *S. cerevisiae*



b) Effect on *K. marxianus*



This suggests that rapid increases in protein and RNA levels in *S. cerevisiae* are another factor in its rapid increase in cell volumes and utilisation of glucose by fermentation. The rise in RNA levels was seen to decrease once the cells were again subjected to a glucose feed of 1g/l glucose, indicating that the original increase was due to increased production of factors involved in the utilisation of glucose. RNA levels also decreased in *K. marxianus* but not to great an extent as observed in *S. cerevisiae* and the cells appeared to recover and continue producing high levels of RNA. It would appear that the increase in glucose levels acts to stimulate anabolic processes as the levels of free amino nitrogen (FAN) in both yeast species remained very low, with apparently little variation.

Results of the effect of chemostat glucose-pulsing on the intracellular carbohydrates, trehalose and glycogen, and the osmolyte, glycerol are illustrated in Figure 4.15. Firstly, trehalose and glycogen respond in an expected manner for both yeast species under these conditions. That is, before the pulse of glucose was applied to the cultures, both yeast species exhibited reasonable levels of both carbohydrates (2% dry cell weight for trehalose and 3-6% dry cell weight for glycogen). When the pulse was applied, the cells reacted with an overall depletion of trehalose and glycogen. In *S. cerevisiae,* glycogen decreased to a level of 0.4% dry cell weight (DCW), where it remained for a time before levels accumulated to about 1.5%. On reverting to 1g/l glucose, the level of glycogen continued to rise for 6h before being completely depleted by 33h. After this depletion glycogen levels appeared to rise back toward steady state levels. Trehalose experienced a 50% increase in response to the glucose pulse before being degraded to a very low level that would appear to represent a basal cellular level

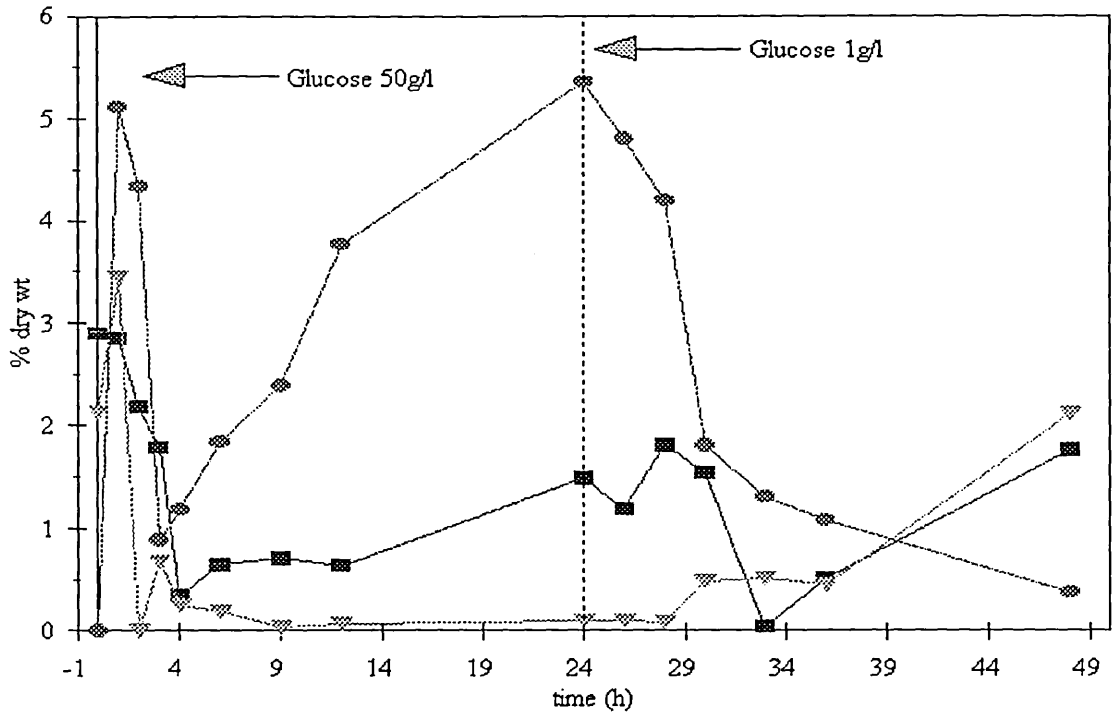
(0.1-0.2%). It was not until 6h after the return to 1g/l glucose in the feed medium that trehalose levels rose to about 0.5%. It is only after glycogen began to return to pre-pulse levels that trehalose did likewise.

In *K. marxianus,* glycogen levels rose dramatically within the first few hours following a glucose pulse, before being depleted to about a sixth of the pre-pulse value. Cellular glycogen levels remained stable until the switch back to 1g/l glucose when glycogen accumulated once more. This behaviour was similar to that seen in *S. cerevisiae*.

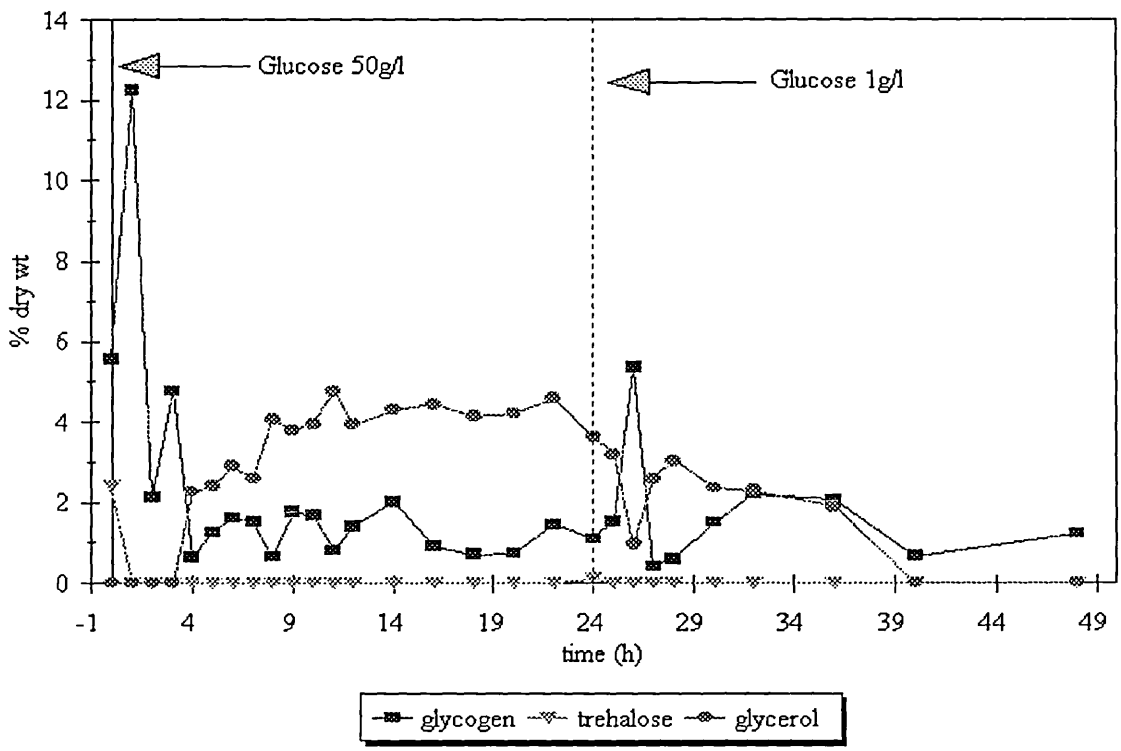
With regard to trehalose in *K. marxianus,* this carbohydrate was completely depleted within 1hr of a glucose pulse, and no apparent recovery was observed through the remainder of the experiment. Trehalose has been described as a stress protectant (see Chapter 1 section 1.6.4) in a variety of conditions that include osmostress. The results presented (Figure 4.15) initially suggested that 50g/l glucose did not constitute an osmotic threat to the cells. However, intracellular and extracellular glycerol levels of both yeast species rose between 4% and 5% of the dry cell weight. There was a difference in the pattern in which each yeast accumulated this compound. As with trehalose, *S. cerevisiae* appeared to respond with a rapid accumulation of glycerol, before it was depleted and then re-accumulated, between 3-24h, to about 5%. When the feed glucose level was changed back to 1g/l, the level of glycerol in the cells rapidly reduced from 5% to about 1.75% in 6h. Glycerol levels continued to fall, but did not reach zero before 48h.

Figure 4.15 Effect of glucose pulse on intracellular levels of trehalose, glycogen and glycerol in yeast growing in chemostat culture at $0.15h^{-1}$.

a) Effect on *S. cerevisiae*



b) Effect on *K. marxianus*



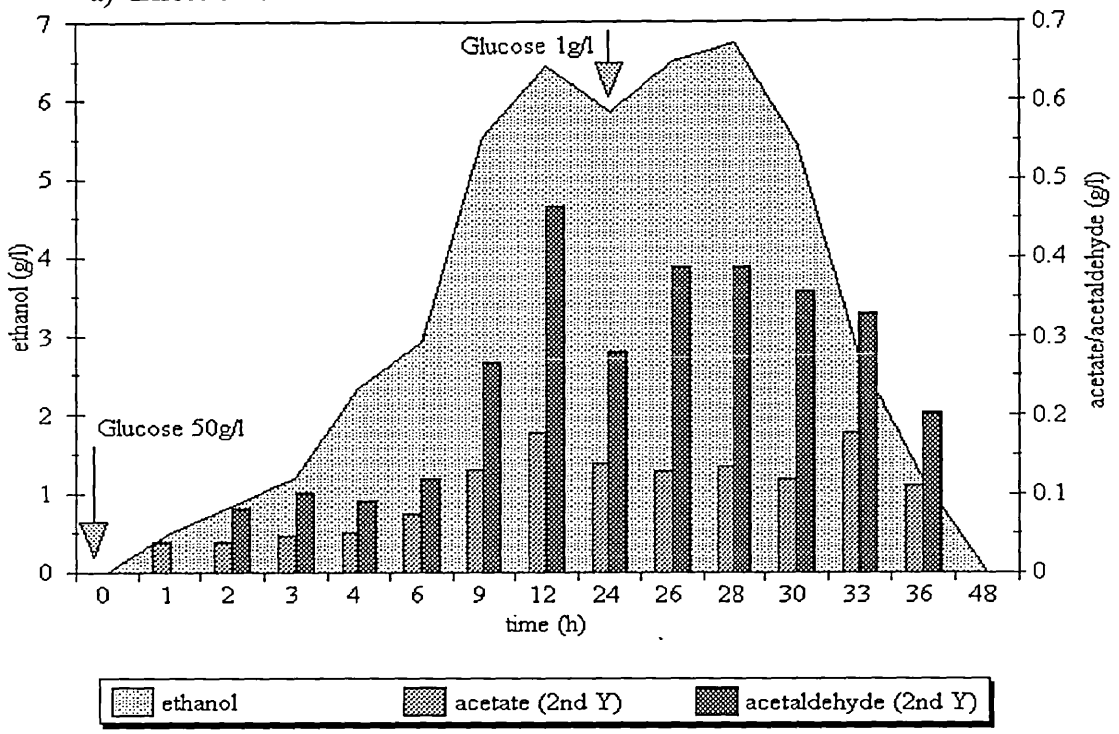
In *K. marxianus,* no accumulation of glycerol was seen until 4h after the glucose pulse, where there was a rapid rise to 2%. The level rose to about 4.5% by 9h and remained at this level during the 50g/l glucose feed. On returning to glucose-limiting feed, the glycerol level within the cells was depleted to zero by 40h.

These results suggest that the production of glycerol is due to osmotic effects affecting both yeasts. In *S. cerevisiae*, there is additional need to recycle NADH to NAD^+ which is required in order that glycolysis may continue. It is clear from the Figures 4.10-4.13, that there was a significant difference between the two yeast species in their metabolic response to the increased glucose concentration.

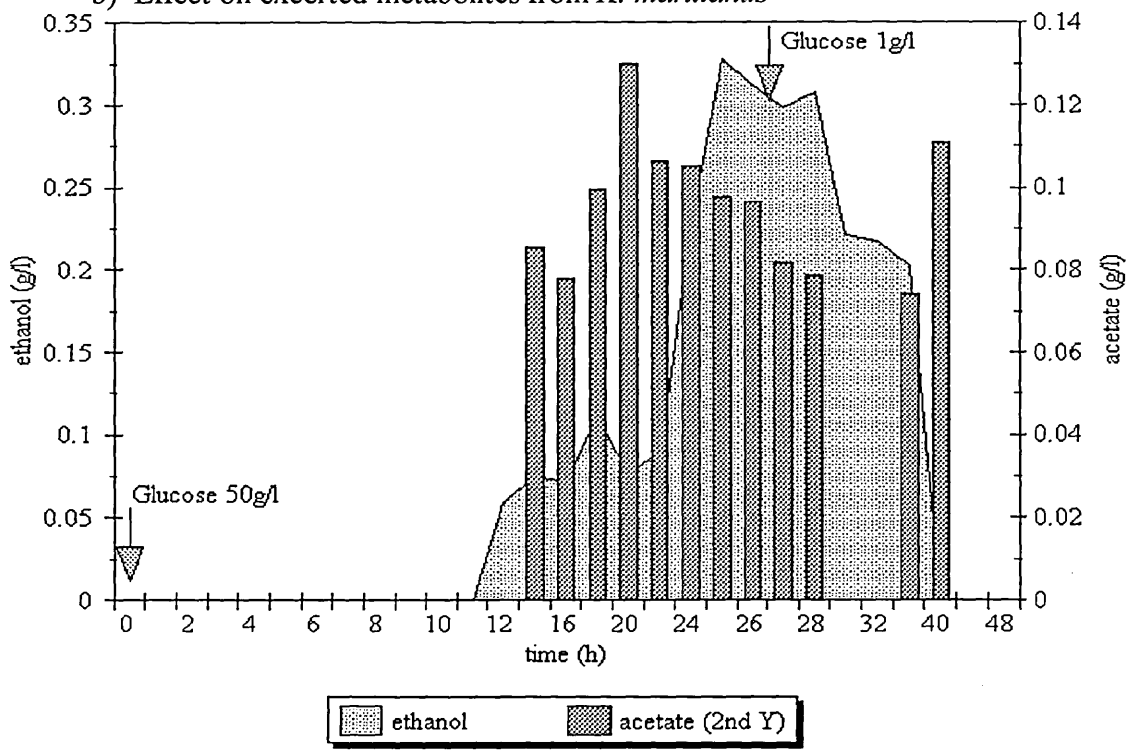
Figures 4.16-4.17 show this more clearly and allow direct comparison of the yeasts' predominant metabolic pathways. Figure 4.16 shows the excretion of the major fermentation products, and in particular those that are most toxic to the cells, i.e. ethanol, acetate, and acetaldehyde. It is noticeable that due to the lesser amount of alcohol produced in the cultures of *K. marxianus* that there is little excretion of either ethanol or acetate. It is conceivable that this small level of fermentative metabolism was not due to oxygen limitation as there was still a significant rate of oxygen uptake occurring in the culture at the onset of ethanol production (about 80mmol/g/h compared with 120mmol/g/h pre-glucose pulse). Postma *et al.* (1989) have suggested that the onset of acetate production occurs before the formation of ethanol in continuous cultures of *S. cerevisiae*.

Figure 4.16 Effect of a glucose pulse on the excretion of major fermentation metabolites from yeast growing in chemostat culture at $0.15h^{-1}$.

a) Effect on excreted metabolites from *S. cerevisiae*



b) Effect on excerted metabolites from *K. marxianus*



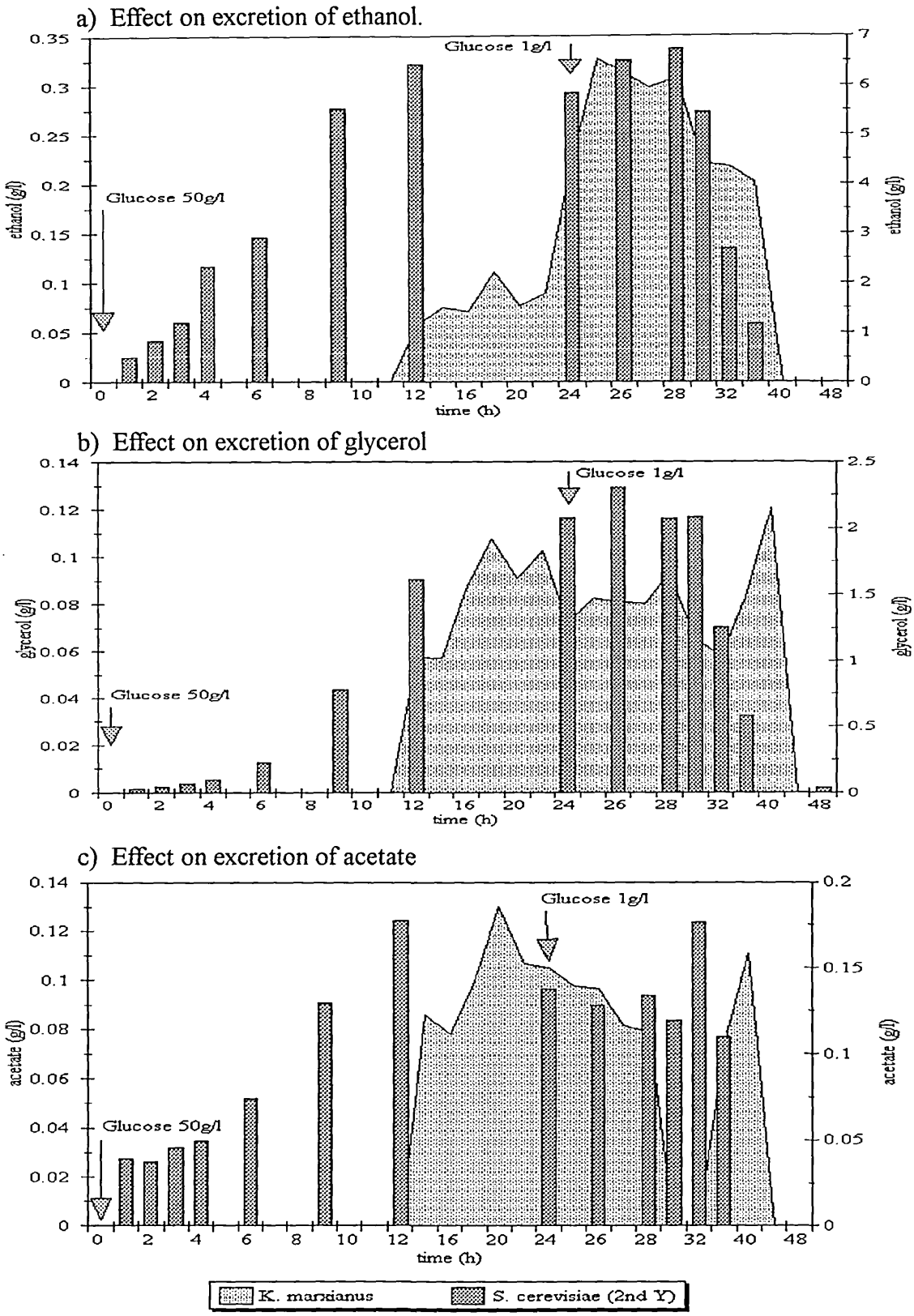
The reason for the onset of fermentation of glucose by *S. cerevisiae* is thought to be due to its limited respiratory capacity (Kappeli and Sonnleitner 1986). Van Hoek *et al.* (1998) suggested that the formation of acetate occured at the same time as the onset of ethanol formation. However, research by the same group has shown that it may be possible for the appearance of acetate to occur before ethanol formation (Van Hoek *et al.* 1998a). What is certain is that *S. cerevisiae* baker's strain GB4918 exhibited significant ethanol production within the first hour of exposure to increased glucose levels and this continued until well after the glucose feed was returned to 1g/l. In *K. marxianus,* ethanol excretion only occurred after 12h with 50g/l glucose in the feed medium. Acetate was detected at the same time as ethanol excretion in *S. cerevisiae* but appeared 2h later than ethanol excretion in *K. marxianus.* The appearance of acetaldehyde in the spent medium of *S. cerevisiae* occurred just 1hr after the detection of ethanol and acetate but not at all in the cultures of *K. marxianus.* This is probably due to the nature of the fermentation in the two yeasts. That is, fermentation is so weak in *K. marxianus* that acetaldehyde never reaches detectable levels, is reduced to ethanol, or oxidised to acetate before it is able to be excreted.

The data presented in Figure 4.17 show the excreted ethanol, glycerol, and acetate by both yeasts. These results make it clear that one of the biggest metabolic differences between these two yeast species appears to be the kinetics of the response to glucose availability. In *S. cerevisiae* this is immediate, whereas in *K. marxianus,* both glycerol and ethanol occur at 12h, whereas acetate appeared 2h later. The almost 20-fold magnitude difference in excretion of both ethanol and glycerol in *S. cerevisiae* compared with *K. marxianus* prove that not only is the fermentative behaviour of *S.*

cerevisiae stronger, but so also is the production of glycerol. The data in Figure 4.15 showed that both yeasts contained the same level of glycerol, but the data in Figure 4.17 shows that *S. cerevisiae* excreted glycerol at levels of 0.6% DCW at every sample point from 12 to 30h.

In comparison, acetate was excreted by both yeasts at similar concentrations suggesting that both yeast species have a very low tolerance to acetate within the cell. It could also mean that both yeast species have a similar capacity for dealing with acetate aerobically. However, in *S. cerevisiae,* this aerobic pathway was repressed by the increased presence of glucose, which resulted in a switch to removing both acetate and acetaldehyde by producing more ethanol.

Figure 4.17 Comparison of excreted metabolites from yeasts during exposure to glucose pulse in chemostat cultures at $0.15h^{-1}$.



Both yeasts may have an active pyruvate dehydrogenase bypass. In this system, pyruvate that is not utilised by mitochondrial pyruvate dehydrogenase is decarboxylated by pyruvate decarboxylase to acetaldehyde. It is at this point that the two yeasts may differ. Once acetaldehyde is produced it can be oxidised to acetate or reduced to ethanol, by the action of acetaldehyde dehydrogenase and alcohol dehydrogenase, respectively. In cells where the predominant metabolism is respirative, acetaldehyde would be oxidised to acetate, and acetate used to form acetyl CoA by the action of the enzyme acetyl CoA synthetase. This enzyme has been postulated as the limiting step in the pyruvate dehydrogenase bypass (Postma *et al.* 1989). When it is saturated this would result in an accumulation of acetate, without ethanol formation. Once the capacity of acetaldehyde dehydrogenase was reached then the cells would have to remove excess acetaldehyde by the activity of alcohol dehydrogenase. This may be the state of the *K. marxianus* cells 12h into the 50g/l glucose pulse. In *S. cerevisiae*, there is the complication of the Crabtree effect and glucose repression, acting on electron transport and oxidative phosphorylation. It has been established that under anaerobic conditions, the mitochondria of *S. cerevisiae* condense into few, large, branched organelles, rather than exist as numerous, small, rounded organelles (Visser *et al.* 1995). It would have been of interest to observe mitochondrial size in these experiments to see if part of the cell volume changes seen in Figure 4.2 are consistent with changes in mitochondrial morphology. It is known that while the mitochondria have no role to play with regard to energy metabolism in fermenting cells that they are not redundant structures in fermenting cells (Visser *et al.* 1994). Fermenting mitochondria are known to play important roles in lipid and amino acid biosynthesis (O'Connor-Cox *et al.* 1996a).

CHAPTER 5 - CELL PHYSIOLOGY OF BAKER'S YEAST IN CHEMOSTATS SUBJECTED TO NUTRIENT PERTURBATIONS

1. Inorganic Effectors.

5.1. Introduction

Chapter 3 described the steady state composition of *S. cerevisiae*, with respect to the macromolecular components, and confirmed that this was dependent on the growth rate at which it was being studied. In the planning of perturbations to the steady state it was apparent that selecting a set growth rate, at which the yeast had already been defined, was necessary in order to investigate any changes to the yeasts' physiological state.

Perturbations were selected which were relevant to both fundamental biological research and to the industrial propagation of yeast. The first perturbations chosen were those involving altered availability of inorganic cations. The main cations of interest were magnesium, calcium and zinc. Previous research by Chandrasena (1997) had shown that altering the levels of these cations in molasses had significant effects on ethanol fermentations by *S. cerevisiae*. The relevance to current research was that improved ethanol production often appeared to correspond with improved cell growth and that the optimisation of media cationic composition may lead to improved yeast biomass yield when propagated under aerobic conditions. The importance of these cations in yeast physiology and metabolism was discussed in Chapter 1.

With this in mind a series of perturbations was developed that could be divided into two areas of the yeast's nutrition. The first area was that of inorganic nutrition, which involved those effectors described in this chapter. The second area was that of organic nutrition and contaminants which will be discussed in more detail in Chapter 6.

In the present case inorganic effectors are taken to be components that supply the yeast's ionic nutritional components, e.g. Ca^{2+}, Zn^{2+}, Mg^{2+}. All of the inorganic effectors studied in this chapter are cationic, and are all metallic in nature.

The focus of current interest was the influence of magnesium and calcium on baker's yeast physiology and macromolecular constituents. Other researchers have suggested that the ratio between magnesium/calcium is as important a consideration in influencing *S. cerevisiae* physiology as absolute concentrations of the two cations present in the yeast's growth environment (Walker *et al.* 1996).

5.2. Experimental Approach

Experiments to investigate the perturbation of the steady state culture of baker's yeast were carried out under the same conditions as described in Chapter 2. The perturbation was initiated by injection of a "spike" of the particular cation of interest, directly into the chemostat. Calculations were carried out to determine the level of the cation present in the chemostat as time passed and the physical effect of washout.

The levels of all three cations (calcium, magnesium and zinc) were calculated and, as discussed in Chapter 1, it was deemed that altering the magnesium level would

have little effect on the state of the yeast as the medium used in this research is already balanced heavily in the favour of magnesium. For example, the Mg : Ca concentration ratio in QEMM3 was 12:1. This suggested that it would be more interesting to study the effect of increased levels of calcium as opposed to increased levels of magnesium. High levels of calcium are a well known problem in molasses used in industrial yeast propagations, not only in terms of inhibiting yeast growth and metabolism, but in the physical problem of "limescale" where calcium salts, especially sulphate are laid down in pipe systems and other processing equipment. Another major factor in this decision was the practical side of adding higher levels of magnesium sulphate to the chemostat in volumes of less than 10ml. Much larger volumes added to the chemostat would almost certainly disturb the steady state. Even at the lower dilution rate used in these experiments ($0.12h^{-1}$) 10ml would account for a residence time of less than 4min, whereas at the higher dilution rate the residence time for the 10ml 'spike' would be just over 2min.

To establish the magnitude of the required spike it was necessary to calculate an average molasses composition for Mg^{2+} and Ca^{2+} and compare this with cation levels in the defined medium, QEMM3. In this way, an attempt was made to simulate cation levels commonly found in industrial fermentation/propagation substrates in a chemically-defined yeast growth medium. The simplest way of doing this was to take an average composition of beet molasses and add it to the average composition of cane molasses and divide this result by 2 (see Table 5.1). This would, by no means, be an accurate representation of molasses medium Mg^{2+}:Ca^{2+} ratios but it would suffice as an example for the purposes of these experiments. In general, the mixture of molasses in commercial

yeast propagations contains more cane molasses than beet molasses, anywhere from 60:40 to 80:20 in favour of cane molasses. This would produce a molasses feedstock that would probably be higher in both Mg^{2+} and Ca^{2+} content than this idealised example.

Table 5.1. Average Mg^{2+}:Ca^{2+} composition in idealised molasses.

(from Walker *et al.* 1996).

	Mg^{2+} (mg/l)	Ca^{2+} (mg/l)	Mg^{2+}:Ca^{2+}
Beet molasses	67	750	0.09
Cane molasses	700	5,000	0.14
Total	767	5,750	0.133
Average	383.5	2,875	0.133

Table 5.2. Levels of Mg^{2+}:Ca^{2+} in QEMM3 before and after perturbation.

Medium Component	Level in medium (mg/l)	Levels after perturbation (mg/l)
Mg^{2+}	98.5	98.5
Ca^{2+}	8.19	819
Ratio Mg^{2+}:Ca^{2+}	12	0.12

The data in Table 5.1 made it clear that the balance in molasses is heavily tipped in the favour of calcium and this has been reported as affecting the fermentation performance of yeast in this medium (Walker *et al.* 1996). When compared with the defined medium, QEMM3, it was clear that while the media were balanced in opposite directions, with regard to Mg^{2+} and Ca^{2+}, there appeared to be only a difference in magnitude, of about 100-fold. The calcium spike that was then chosen (810.81mg/l) would be used to redress the balance of calcium in QEMM3 to that similar in molasses media.

It was more difficult to decide upon a Zn^{2+} perturbation as this cation can be toxic to yeast cells under certain conditions (Jones and Greenfield 1984). However, at trace levels it has an essential role in yeast metabolism and physiology, particularly in the activity of alcohol dehydrogenase and in the structure and function of DNA. In QEMM3, Zn^{2+} is present at 0.8mg/l as zinc sulphate heptahydrate which means that only 0.182mg/l of Zn^{2+} is present. This is slightly below the range of zinc required for optimal yeast growth reported (4-8μM) by Jones and Greenfield (1984), although optimum concentration for growth can be as low as 0.1-0.3μM.

Table 5.3. The Zn^{2+} and Mn^{2+} content of QEMM3.

	Level in medium	After perturbation
Zn^{2+} (mg/l)	0.182	0.91
Zn^{2+} (μM)	2.78	13.9
Mn^{2+} (μM)	10.92	10.92

The data in Table 5.3 (Jones and Greenfield 1984) shows both the Zn^{2+} and Mn^{2+} content of the medium and the molarity of the metal ions. The information regarding Mn^{2+} is included due to its synergism with Zn^{2+}. When Mn^{2+} is present in the medium at concentrations above 7μM then it appears to offset the toxicity of Zn^{2+} which normally occurs at 30μM when Mn^{2+} is absent or below 7μM (Jones and Greenfield 1984). Mn^{2+} is also required by the yeast although it has few metabolic roles for which it can not be substituted.

5.3. Results

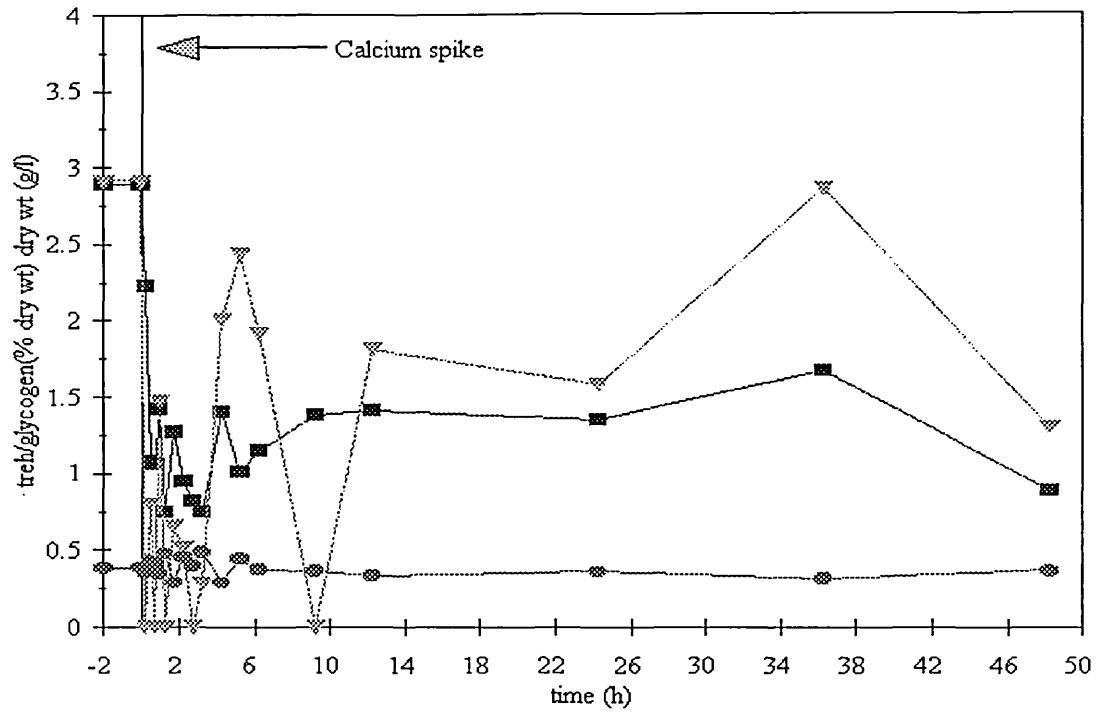
Results showing the effects of both calcium and zinc perturbations on the steady-state growth of *S. cerevisiae* at two growth rates are shown on Figures 5.1-5.4. Sub-*optimal* growth rate ($0.12h^{-1}$) results are represented in the upper part of Figures and *supra-optimal* growth rate ($0.22h^{-1}$) results represented in the lower parts.

The effect of a sudden elevation of calcium ion concentration to 819mg/l in the chemostat (that is, a "calcium spike") is shown in Figures 5.1 and 5.3. Figure 5.1 shows the effect of a calcium spike on changes in biomass and storage carbohydrates of *S. cerevisiae*. In the case of yeast biomass there is little effect of elevating calcium levels at either of the two growth rates. However, the effect on both glycogen and trehalose is more significant. With glycogen, there appears to be a very rapid degradation of the intracellular levels (within 15min) and cells do not return to steady state levels observed prior to initiation of the calcium spike. This was observed at sub-optimal growth rates but not under super-optimal conditions where the level of glycogen apparently recovers to near steady state levels before a further period of degradation followed by another small recovery.

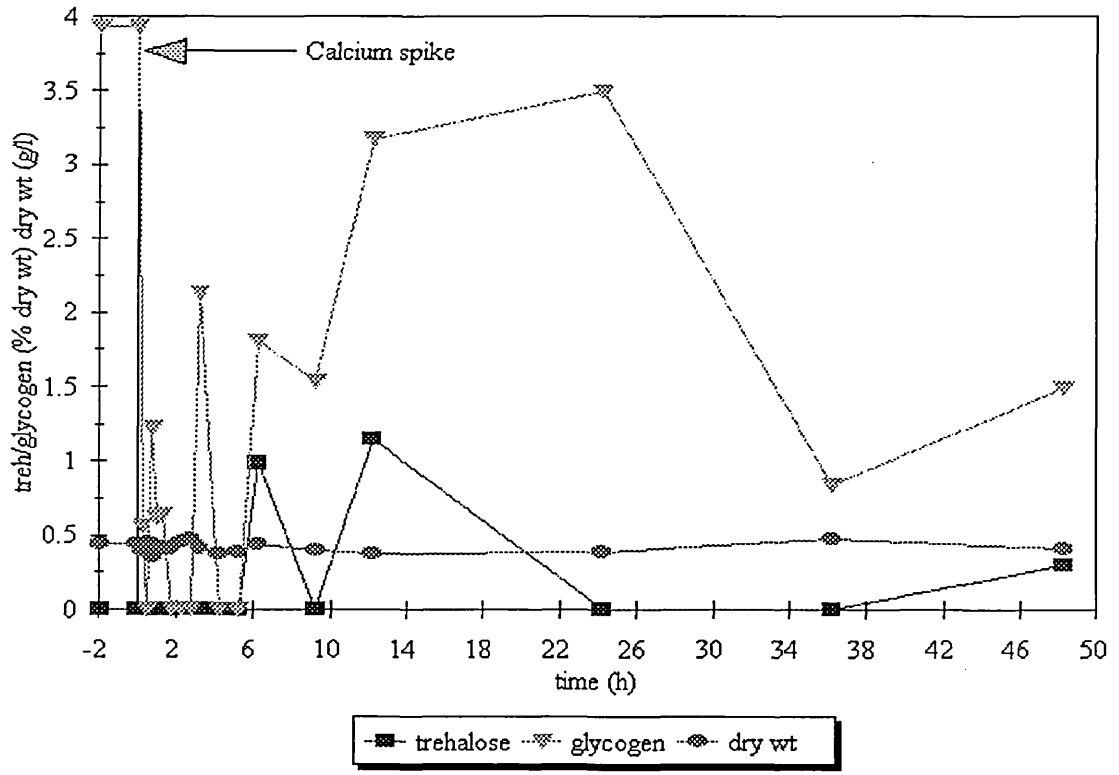
Yeast trehalose levels responded differently to that of glycogen in that this storage disaccharide was primarily degraded to about a third of it's steady state level at sub-optimal growth rate. Trehalose levels eventually recovered to a value just half of that of the steady state. At the supra-optimal growth rate trehalose was undetectable in the cells until 6h after the calcium spike was introduced. This level fluctuated for the following 6hr before returning to zero at 24h and rising again only slightly by 48h.

Figure 5.1 Effect of a calcium spike on yeast storage carbohydrates. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



b) Supra-optimal growth rate, $0.22h^{-1}$



The synthesis of trehalose involves the enzymes, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase that both have a prerequisite requirement for Mg^{2+} ions. It is known that in many biological systems there is an antagonistic relationship between Mg^{2+} ions and Ca^{2+} ions (Walker 1999). This may be why trehalose is lost at the sub-optimal growth rate of *S. cerevisiae*, as accumulation of trehalose is caused by a shift in the balance of its anabolic/catabolic reactions (Salek and Arnold 1995); (San Miguel and Arguelles 1994) The major catabolic enzyme of trehalose is the cytosolic or neutral trehalase and it has a requirement for Ca^{2+} or Mn^{2+}. This would mean that in the chemostat, during the steady state, conditions may favour the anabolic reactions, due to the medium bias for Mg^{2+} over Ca^{2+}.

It is unclear from previous literature what the precise cationic requirements are for the glycogen metabolic pathway, but as the anabolic pathway involves phosphorylases then there is probably a requirement for Mg^{2+} in the process. Phosphoglucomutase, the enzyme responsible for production of the important glycogen building block, glucose-1-phosphate from glucose-6-phosphate, has a requirement for Mg^{2+}. It also known that UTP-glucose-1-phosphate uridyltransferase also requires Mg^{2+}, as this is a phosphate related reaction it is also possible that the ATP-form of this enzyme also requires Mg^{2+}. However, it is unclear if glycogen synthase has a requirement for either of the cations, Mg^{2+} or Ca^{2+}. It is known that the control of the enzymes involved in glycogen metabolism is very tightly regulated by their state of phosphorylation, this is especially true for glycogen synthase and glycogen phosphorylase (Clotet *et al.* 1995; Lin *et al.* 1995; Thon *et al.* 1992). In the metabolism of glycogen in mammals, calcium is known to act as an activator in the control of enzymes that are involved in the cyclic

cascade that controls the synthesis and degradation of glycogen (Figure 5.2). Calcium is known to act in favouring the activation of the glycogen degrading enzyme, glycogen-phosphorylase (Lin *et al.* 1995), while influencing the de-activation of glycogen synthase. The overall effect of increasing availability of calcium in the yeast chemostat culture would be the degradation of glycogen.

Figure 5.2 A schematic diagram of the enzymatic modification/demodification systems involved in the control of glycogen metabolism (adapted from (Voet and Voet 1990))

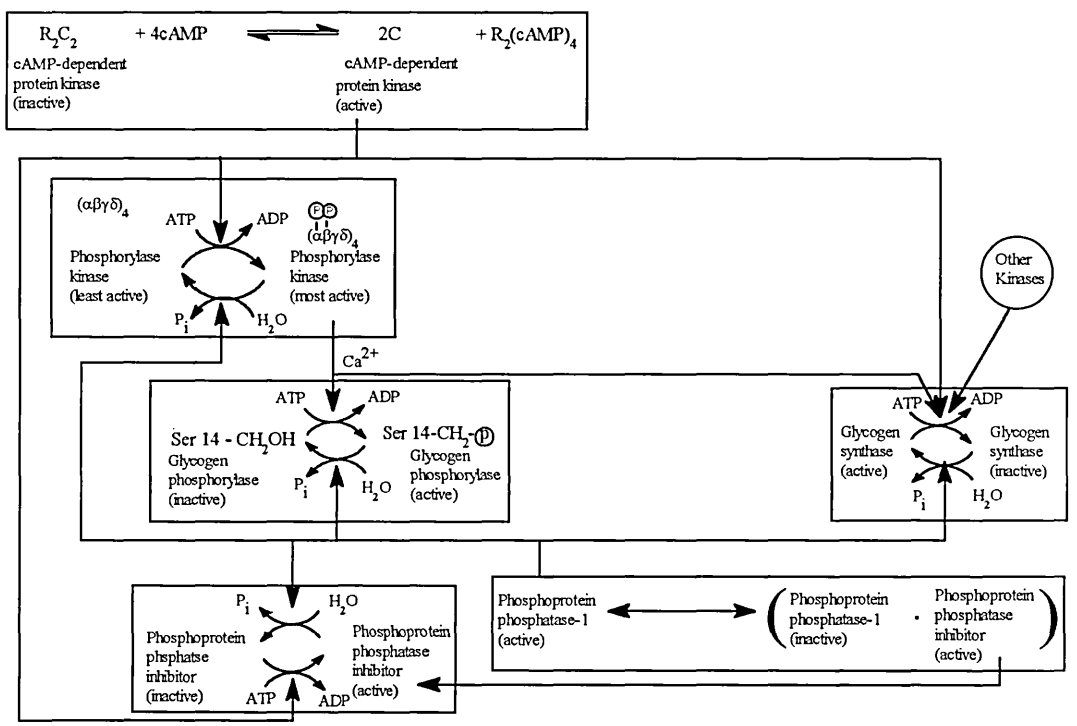
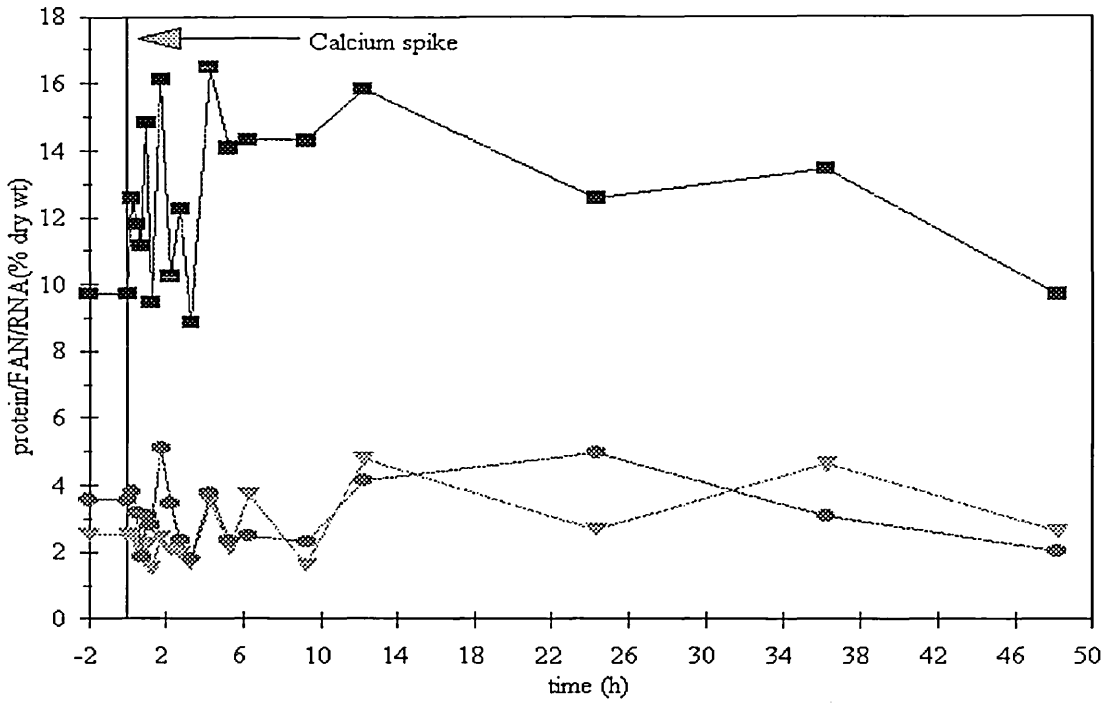


Figure 5.3 Effect of a calcium spike on yeast protein, FAN, and RNA. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



b) Supra-optimal growth rate, $0.22h^{-1}$

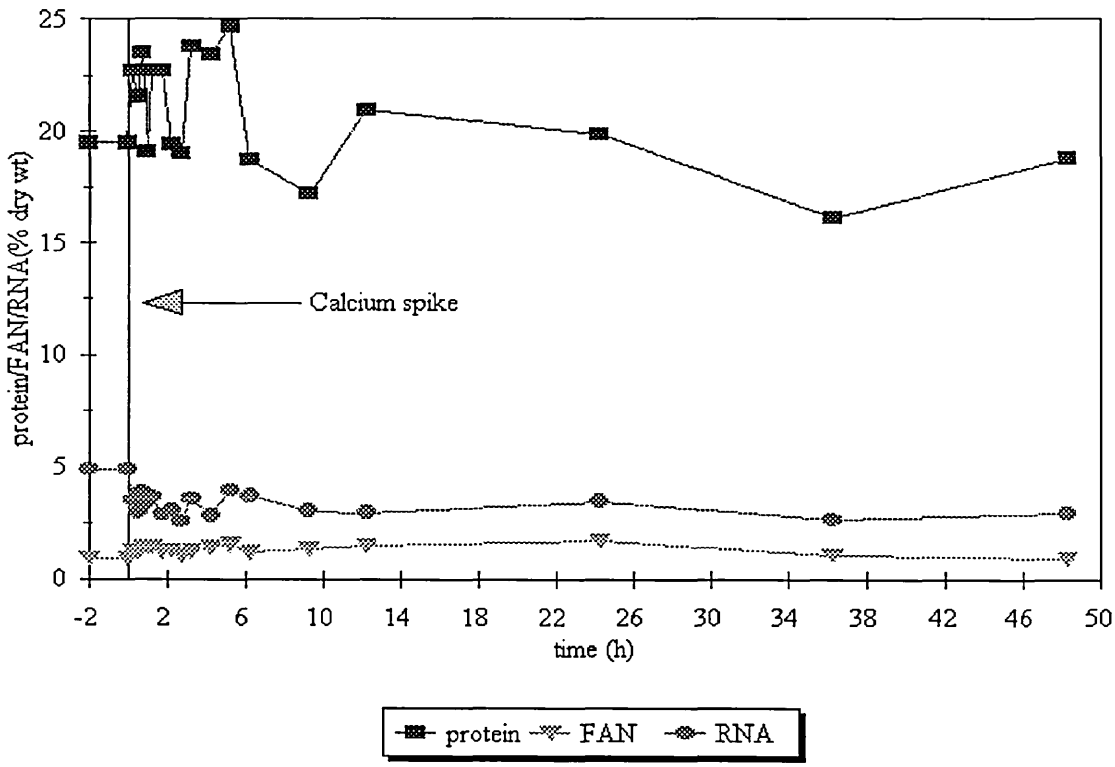


Figure 5.3. shows the metabolism of the nitrogen compounds that were analysed and it is clear that elevating calcium levels allowed the cells to increase their protein levels at both growth rates studied. The differences between the responses of *S. cerevisiae* appear to be due to the specific growth rate, with the cellular level of protein returning to steady state levels after 48h. In the supra-optimal culture the elevation in protein levels lasts for less than 12h. Protein levels are seen to be higher in the cells grown at the higher growth rate, a trend that was also recorded in the characterisation of the cells at the steady state discussed in Chapter 3. The supra-optimal growth rate used here is below that at which protein was lost due to the metabolic switch from respiration to respiro-fermentative in *S. cerevisiae* (ultimately, washout).

It would appear that the effect of the calcium spike on FAN is also a slight elevation in levels that appears to be growth rate sensitive. Thus, the elevation persists at sub-optimal growth rate and appears to disappear very quickly at supra-optimal growth rate. Interestingly, the calcium spike did lower the intracellular levels of RNA in both culture conditions, and neither appears to recover to steady state levels during the term of the experiments. There is a rise in RNA levels in the sub-optimal culture between 12-36h, followed by a reduction in levels to that seen immediately following the calcium spike.

The increase in protein levels may be due to increased metabolism of cellular components that are not measured here, especially those involved in the cell membrane. The formation of the key sterol, ergosterol, is known to have associations with calcium

(Crowley *et al.* 1998). Calcium is though to be a non-essential cation with regard cell growth and metabolism, but there does exist a need for calcium within the yeast cell as has been reported for some physiological responses by yeasts, hypotonic shock (Batiza *et al.* 1996). It has also been suggested that yeasts actively exclude calcium, as well as sodium ions from the cell by processes of compartmentalisation and plasma membrane calcium transporters. Calcium also has a specific binding protein, called calmodulin, that acts to 'mop-up' cytosolic calcium and tightly regulate its intracellular free concentration at sub-micromolar levels. Calcium has also been described as a blocker of magnesium in certain enzymes, especially those that are to be transported in an inactive form to the extracellular environment (Walker 1994). It has also been well documented that calcium can inhibit growth of yeast cells at concentrations above 25mM (Jones and Greenfield 1984) and that a fermentation medium unfavourably balanced for calcium against magnesium similarly reduces the ability of yeast to ferment effectively (Walker *et al.* 1996);(Walker *et al.* 1994b).

Figure 5.4 shows the effects of elevating zinc levels to 14mM on yeast biomass, trehalose, and glycogen. For both sub- and supra-optimal growth rates, the effect of a zinc spike was quite similar and appeared to be independent of growth rate. Both cultures are seen to lose biomass, very slightly, for the first 6h before a recovery is seen. Further variations in biomass after this recovery are probably due to the disturbance in the steady state. The effect of the zinc spike on trehalose appears to be to degrade the carbohydrate at both growth rates, with a recovery not seen during the 48h of the experiment.

Figure 5.4 Effect of a zinc spike on yeast storage carbohydrates. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

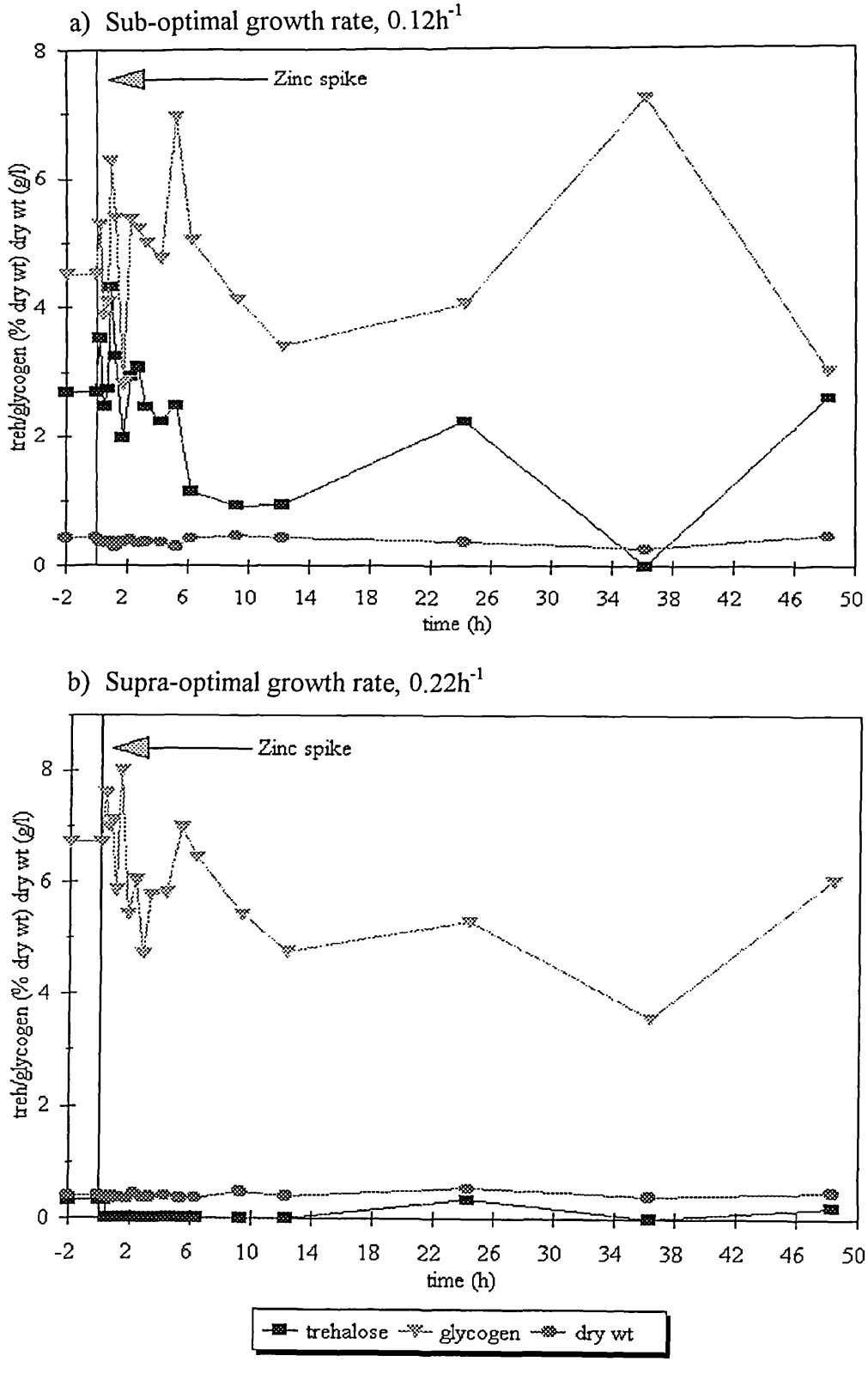
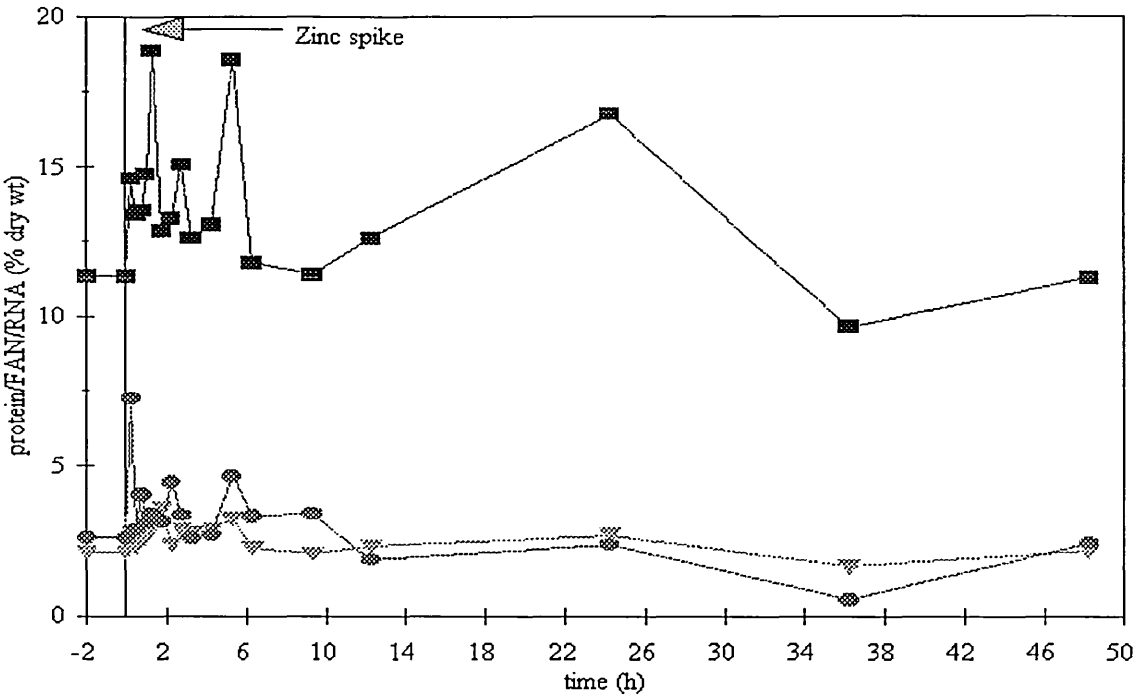
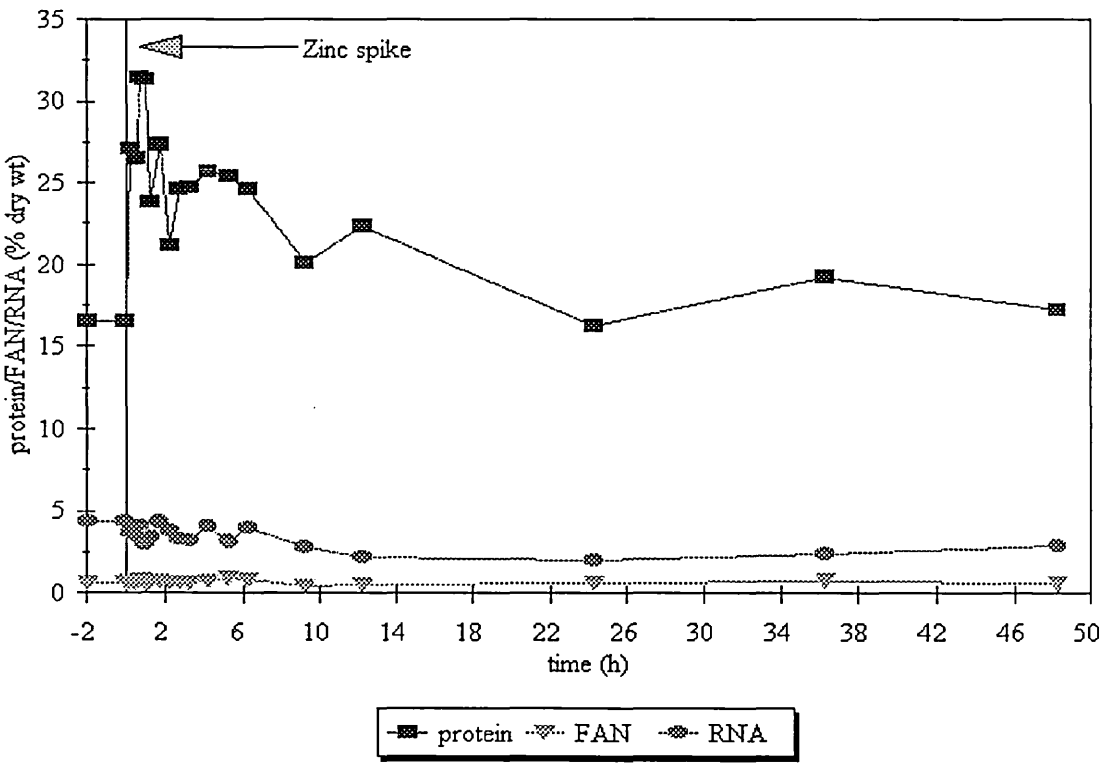


Figure 5.5 Effect of a zinc spike on yeast protein, FAN, and RNA. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



b) Supra-optimal growth rate, $0.22h^{-1}$



Glycogen levels in *S. cerevisiae* appear to react slightly differently at low and high yeast growth rates. In the sub-optimal culture, levels appear to oscillate before the cells appear to return to near steady state levels, although a very high level of glycogen is present at 36h. In the supra-optimal culture, the level of glycogen is reduced by the zinc spike, after a period of oscillation.

There is little scope for any direct effect of zinc on any of these parameters as the levels of zinc that cells were subjected to should be non-toxic due to the high level of manganese also present in the medium (Table 5.3). Enzymes involved in trehalose metabolism do not require zinc as a co-factor, and as stated earlier, enzymes of glycogen metabolism are probably more in need of magnesium. The reason for the changes in the intracellular levels of these carbohydrates is probably due to knock-on effects from other metabolic pathways in the cell, or possibly due to reduced need for the carbohydrates due to the increased presence of zinc ions, or the roles that zinc may play in stimulating the production of cellular factors that had, prior to the spike, been limiting, eg. enhanced riboflavin synthesis (Jones and Greenfield 1984).

Protein levels (Figure 5.5) in both sub- and supra-optimal cultures rise, although there appears to be no effect on FAN in either culture, but a slight raise in RNA levels in the sub-optimal culture. This is consistent with reports that have suggested a link between protein content and zinc levels in fermenting yeast cells (Jones and Greenfield 1984), and cells subjected to a zinc deficiency (Obata *et al.* 1996). Obata *et al.* (1996) observed a very clear correlation between zinc deficiency and protein levels in *S. cerevisiae*; not just in reduced protein levels, but also in patterns of protein expression, in

that some proteins were not expressed in the zinc-deficient cells, but were in the presence of zinc, and vice versa. It is interesting to note that Obata *et al.* (1996) found that one of the proteins that was affected by the presence of zinc was enolase, a key enzyme in glycolysis that does require magnesium, but zinc and manganese can adequately substitute for this cation. The changes to this enzyme were not in its expression but rather in its physicochemical properties with a change in it's isoelectric point, although it is not clear how zinc ions could change this property of a protein.

5.4 Effect of magnesium on trehalose and glycogen metabolism

The results presented earlier in this chapter suggested quite clearly that the extra calcium available to the cells was acting to decrease the levels of both trehalose and glycogen. This has been explained as being due to the stimulatory and inhibitory effects of calcium on the control of degradative and biosynthetic enzymes, respectively, in glycogen metabolism. Whether or not the same is true of trehalose metabolism is unclear, as so many more control factors appear to influence trehalose metabolism, e.g. heat shock, glucose repression. What is certain is that the anabolism of trehalose requires magnesium and the catabolc process requires, calcium or manganese (Thevelein 1996). This warranted further investigation, especially with regard to improving the resistance of baker's yeast to stress.

In order to do this, yeast strains deficient in trehalose accumulation were acquired from the laboratory of Bruce Futcher (Cold Spring Harbor, USA). These strains of *S. cerevisiae* (RTF1.5-2 and RTT 102) were used to study the effect of magnesium on trehalose and glycogen accumulation and the resistance of these cells to

heat shock. The *RTT* mutant is a strain of *S. cerevisiae* that has a null mutation introduced into the gene that encodes for trehalose-6-phosphate phosphatase, *RTF 1.5-2* is the parent strain from which *RTT 102* was obtained. The mutation in the *tps2* gene allows the cell to grow on glucose as a C-source, as in the wild-type strain, whereas a mutation in the *tps1* gene, which encodes the trehalose-6-phosphate synthase enzyme, abolishes the ability of the cell to grow on glucose and fructose (Elliott *et al.* 1996).

5.4.1 Experimental approach

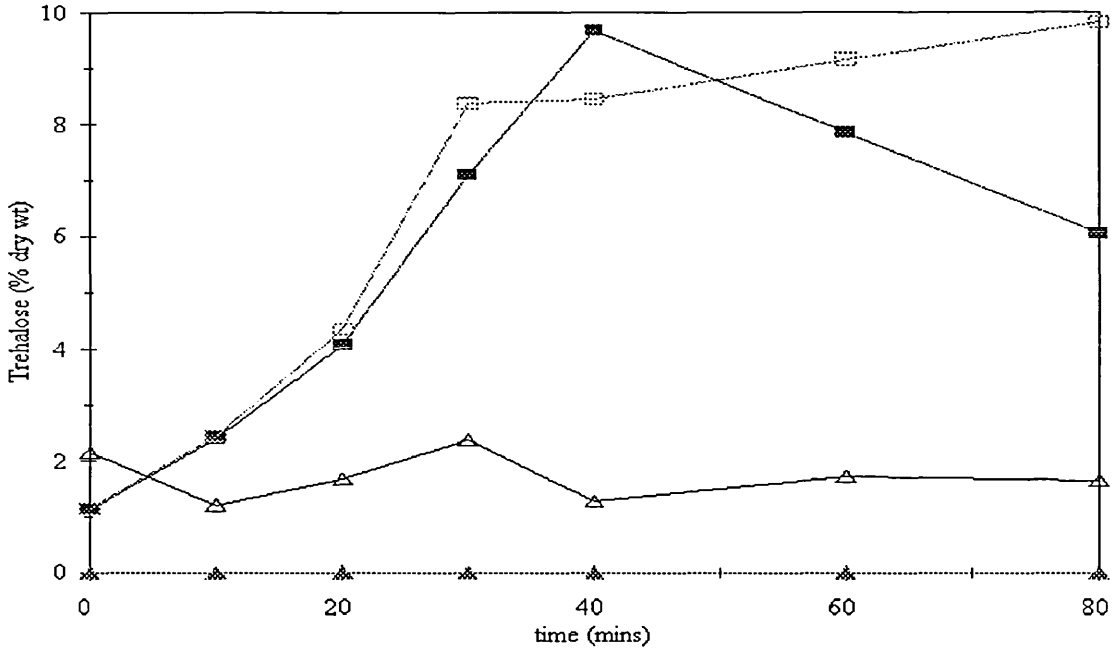
The yeast strains described above were grown in 100mls yeast extract (2%) peptone (1%) (YEP), 2% glucose, some with 40mM added magnesium sulphate, until they had reached stationary phase. The cells were then centrifuged and washed before a known cell number was inoculated into 10 tubes containing 11ml YEP. These tubes were then placed in a water bath at 49°C (heat shock temperature), and a tube was removed every 10 minutes for analysis. The contents of each tube were split into two aliquots, one of 1ml and one of 10ml. The 10ml aliquot was halved and subjected to analysis for trehalose, glycogen and dry weight as descirbed in Chapter 2. The 1ml aliquot was serially diluted, with YEP, and 100µl of the three most appropriate dilutions (generally 10^{-3}, 10^{-4} and 10^{-5}) were plated, in triplicate, on YEPG agar plates. Plate counts were carried out after 48h incubation at room temperature and viability was calcualted against control samples that had not been heat shocked representing 100% viability.

5.4.2 Results and Discussion

Data obtained from these experiments are shown in Figure 5.6 and 5.7 and support the hypothesis that magnesium is required for the synthesis of trehalose. In Figure 5.6 a) it was clear that the extra magnesium had allowed the wild type yeast strain to accumulate trehalose beyond 40min. In the wild type without extra magnesium the cells exhibited a decrease in trehalose levels after 40min. These results also show that increased trehalose accumulation was consistent with increased viabilty, as shown in Figure 5.7. The *RTT 102* strain that is unable to synthesise trehalose reacts as expected when grown in non-supplemented YEPG, in that it accumulated almost no trehalose and that this lack of trehalose resulted in poor viability at 49°C. Interestingly, when this mutant strain had been grown in the presence of extra magnesium it had both improved viabilty and trehalose accumulation behaviour. Moreover, this trehalose accumulation was not initiated by the heat shock as was seen in the parent strain, lending evdence to the existence of an alternative, ADP-glucose dependent trehalose-6-phosphate synthase (Thevelein 1996). The genes described in trehalose biosynthesis are known to be UDP-glucose dependent. The existence of a second set of trehalose biosynthetic enzymes has been argued due to the neccessity for trehalose in sporulating yeast cells, and that classic trehalose-deficient strains appear to sporulate normally (Ferreira and Panek 1993).

Figure 5.6 Effect of increased magnesium availability on the intracellular accumulation of trehalose and glycogen in yeast strains with a mutation in trehalose biosynthesis enzyme, trehalose-6-phosphate phosphatase.

a) Effect of increased magnesium availability on trehalose accumulation



b) Effect of increased magnesium availability on glycogen accumulation

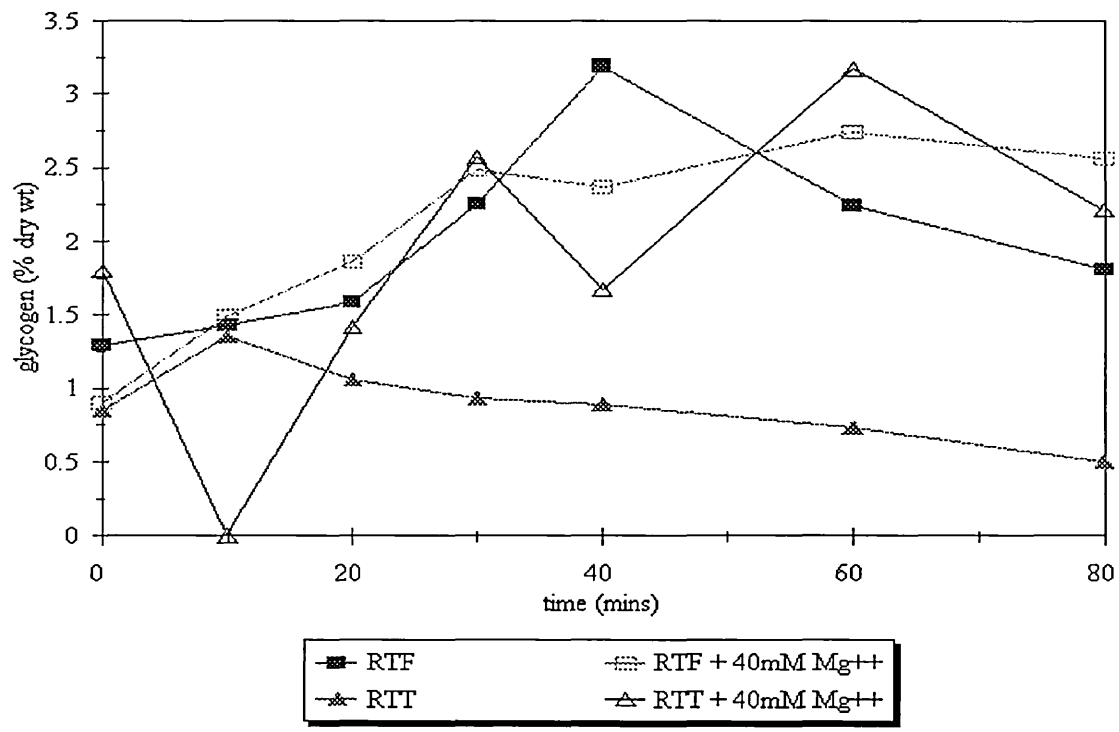
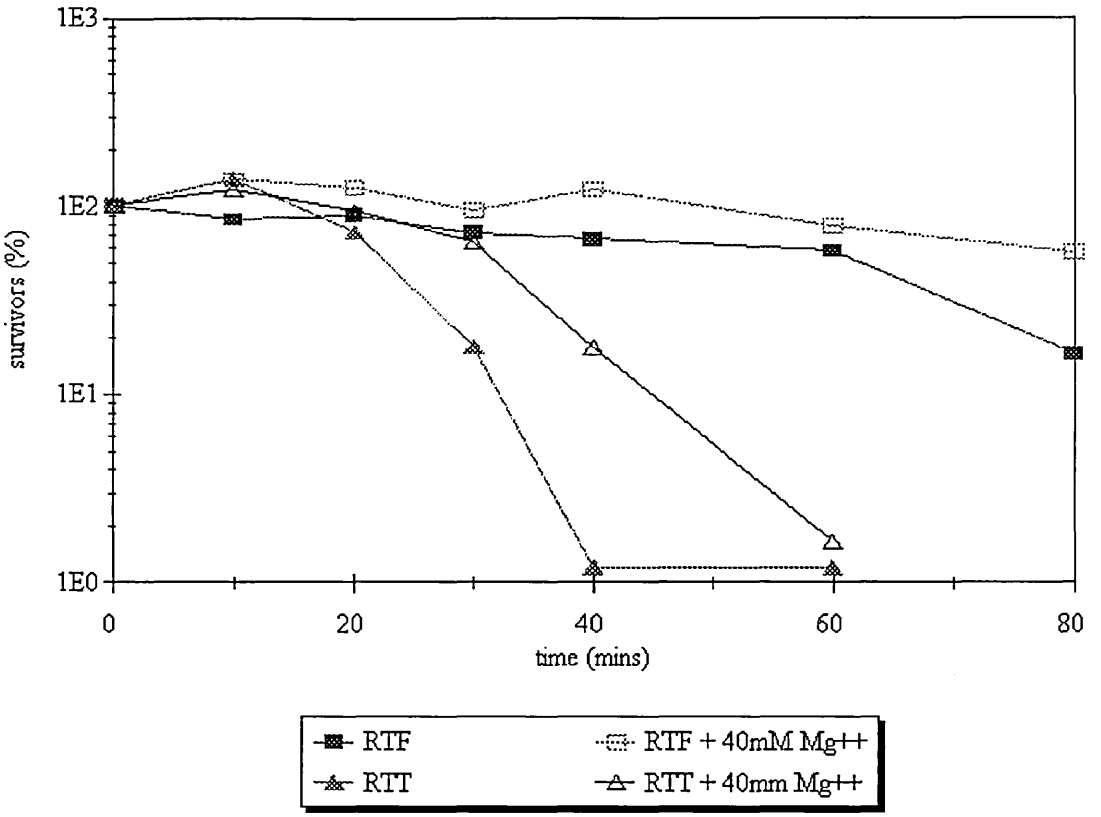


Figure 5.7 Effect of increased availabilty of magnesium on the viability of yeast strains with a mutation in trehalose biosynthesis enzyme, trehalose-6-phosphate phosphatase



In the presence of extra magnesium it appears that glycogen metabolism reacts similarly to trehalose. Only the *RTT 102* strain appeared unable to continue glycogen accumulation beyond 10 minutes and this was probably due to the loss in viability of this yeast strain at that time. In the magnesium-supplemented *RTT 102* strain, the variability in glycogen levels was quite unusual, and difficult to explain, although the overall trend was accumulation of the carbohydrate. In the parent strain, *RTF1.5-2*, there was only a prolonged maintenance of the glycogen levels that was consistent with the improved viability of the yeast when grown with extra magnesium.

These results do provide evidence that increased magnesium availability favours the anabolism of trehalose and that this allows the cells to survive longer under conditions of lethal heat shock. This correlates well with the results from the chemostat experiments where extra calcium acted to reduce trehalose and glycogen levels by favouring the catabolism of these carbohydrates.

CHAPTER 6 - CELL PHYSIOLOGY OF BAKER'S YEAST IN CHEMOSTATS SUBJECTED TO NUTRIENT PERTURBATIONS

2: Organic effectors.

6.1. Introduction

Inorganic effectors were studied in the previous chapter and the main focus of the current chapter is on the action of organic effectors. Organic material in the feedstocks of commercial yeast propagations is derived from the molasses, or sugar cane/beet residues. These can include the sugars that remain after refining and vitamins that survive the various heat treatment stages. Other sources of organic material in molasses may occur through the latter stages of transportation and storage of this raw material. The major problem in this respect is the wild variability in molasses constituents from batch to batch and season to season. Differences in transportation can cause the same supply to differ depending on the respective methods of transport and storage. Due to the high sugar content of molasses it is generally protected from contamination but this does not mean that molasses can not become contaminated. The majority of the contaminants found in stored molasses are located on the surface, due to the high sugar concentration acting as a preservative and the availability of oxygen. The viscosity of the molasses also acts as a good barrier towards microbial activity although it should be noted that molasses is not strictly an inert substance, since it has been reported as reacting extremely violently when it degrades in a particular manner. This is described as "froth fermentation", which is believed to be a wholly chemical process that can result in quite vigorous decomposition of molasses and has lead to the storage of molasses at cooler temperatures (Chen and Meade 1985a). Despite its high sugar content, molasses

is still acted upon by some microorganisms, most of which are probably common to the source of the molasses (Chen and Meade 1985b). This includes some *Lactobacillus* type bacteria which can produce significant levels of lactic acid in the molasses. *Acetobacter sp.* may also grow on molasses and provide small levels of acetic acid. More commonly organisms that grow on molasses are of the osmotolerant type, e.g. xerophilic yeasts like *Zygosaccharomyces rouxii*, which will adapt the environment for contamination by other microflora especially other yeasts.

This makes it difficult for the yeast producer to continue producing yeast of constant quality, despite the requirements of individual customers. One of the variable medium parameters that is studied here is the effect of lactic acid on yeast growth. Lactic acid can occur in molasses naturally but may also accumulate through lactic bacteria during storage and shipping if conditions exist to allow this to happen. Due to the nature of the process involved in growing the yeast it is possible to reduce overall yield of the yeast by simply dosing them with relatively significant levels of lactic acid that may be present in the molasses feed. Reduced yield in an aerobic fed-batch cultivation of yeast tends to signal that energy has been used by the cells for some purpose other than biomass production.

The aim of this research was to study the effect of lactic acid on the physiology and metabolism of baker's yeast strain of *Saccharomyces cerevisiae* grown in an aerobic chemostat with a glucose-limited defined medium.

6.2. Experimental approach

A culture of baker's yeast (*S. cerevisiae* GB4918) supplied by Quest International, Menstrie was grown in a chemically defined medium in a chemostat operating with glucose-limitation at a sub-optimal growth rate, $0.12h^{-1}$ and a supra-optimal growth rate, $0.22h^{-1}$. The cells were allowed to reach a steady state, observed by measuring cell numbers, budding index, biomass and residual glucose levels. Steady state was achieved after 4 vessel volumes. After two control samples were collected, a "spike" of lactic acid, which raised the vessel level to 2% v/v lactic acid, was added aseptically. Samples were collected from the vessel overflow line for periods of 15min for the first hour, then every half hour for 2 hours, and then hourly. Samples were analysed in at least duplicate for dry weight, trehalose, glycogen, protein, free amino nitrogen (FAN) and RNA.

6.3. Results and Discussion

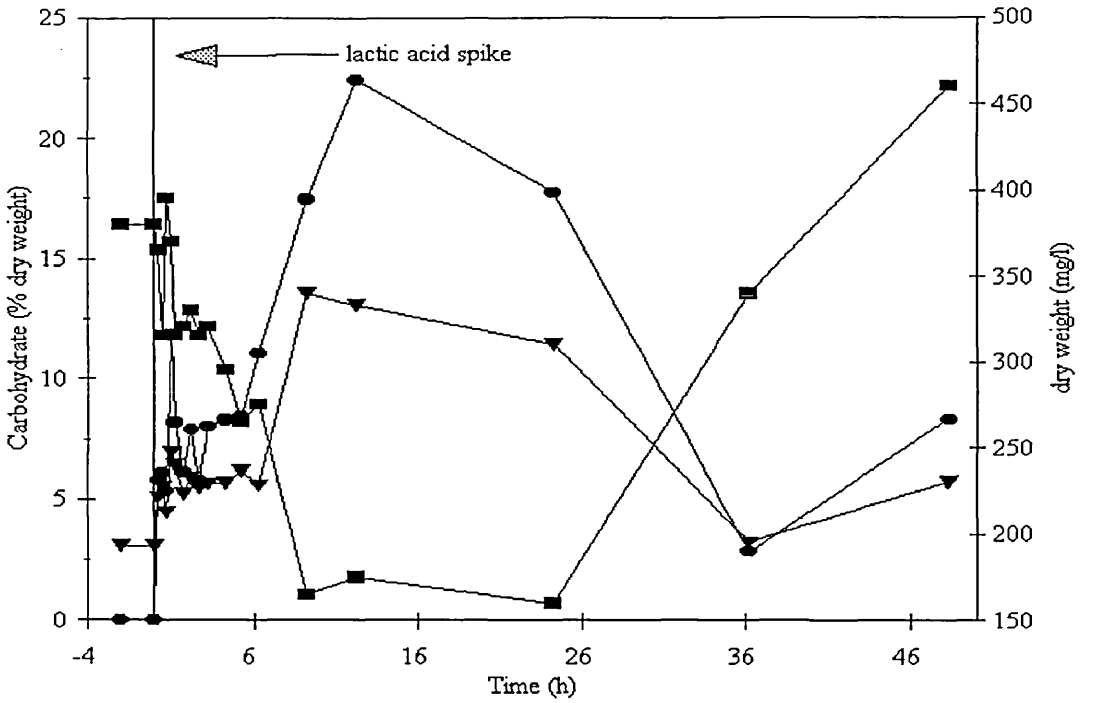
The results presented here represent duplicate cultures at two different growth rates, at $0.12h^{-1}$ termed here as *sub-optimal* and at $0.22h^{-1}$ termed *supra-optimal*. In the case of the supra-optimal growth rate, it should be noted that this culture was still operating below the respiratory capacity of *S. cerevisiae*. Under the conditions of growth used to cultivate this strain of baker's yeast the dilution rate which switched the yeast from entirely respiratory to respiro-fermentative was ascertained to be $>0.24h^{-1}$, this figure is well within the reported range of critical dilution rates for the switch between purely respiratory and respiro-fermentative metabolism (Van Hoek *et al.* 1998b).

From the trends displayed in Figure 6.1, it is clear to see that the major effect of a lactic acid spike was on yeast dry weight with biomass reducing to 45% of the steady state within 10h, where it remains until after 24h when a recovery process began and the biomass overshot to 360mg/L, which was consistent with the steady state. This overshoot was resolved by 72h (data not shown). The recovery of the yeast biomass appeared consistent with the drop of extracellular lactic acid concentration below 0.5ml/vv.(vv = vessel volume). This effect on yeast dry weight was constant in both the sub-optimal and super-optimal growth experiments, although the recovery in the supra-optimal culture was later in occurring and has not overshot in the same way as the sub-optimal culture.

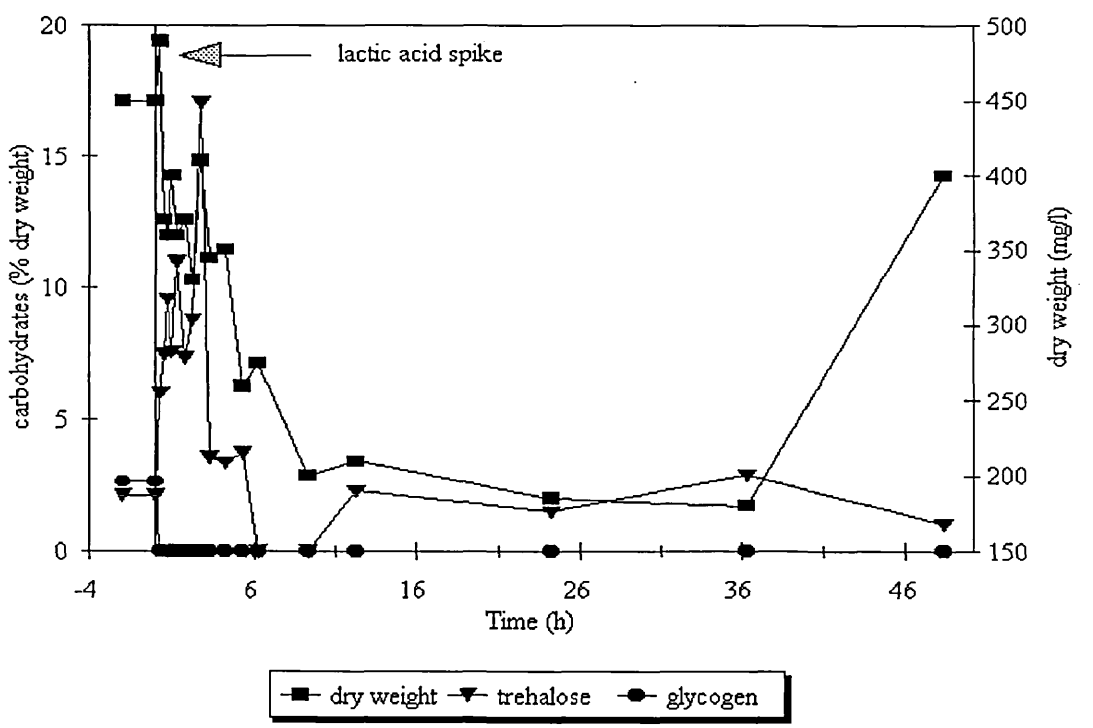
The most dramatic event that occured to the sub-optimally grown cells was the large amounts of glycogen which accumulated. Under normal, aerobic, steady state conditions the cells contained almost no glycogen but within 1h the level was ~7.5% of cell dry wt and this continues to increase to ~27.5% of cell dry wt at 12h. This glycogen was quickly utilised, falling to ~17.5% dry wt by 24 h where the dry weight recovery began. After this, the levels continued to fall and were seen to fluctuate up to 48h. The amount of glycogen present in the 72h sample was not determined. The same trend was noticed with the stress carbohydrate, trehalose, although it only accumulated to ~17.5% from an initial level of ~2.5%. At this dilution rate trehalose was present in yeast, possibly due to the carbon-limiting factor of the chemostat. Under supra-optimal growth these results were clearly not duplicated. Glycogen was not accumulated at all and the levels of trehalose oscillated in the early hours but eventually returned to a pre-perturbation level at 12h.

Figure 6.1 Effect of a lactic acid spike on yeast storage carbohydrates. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



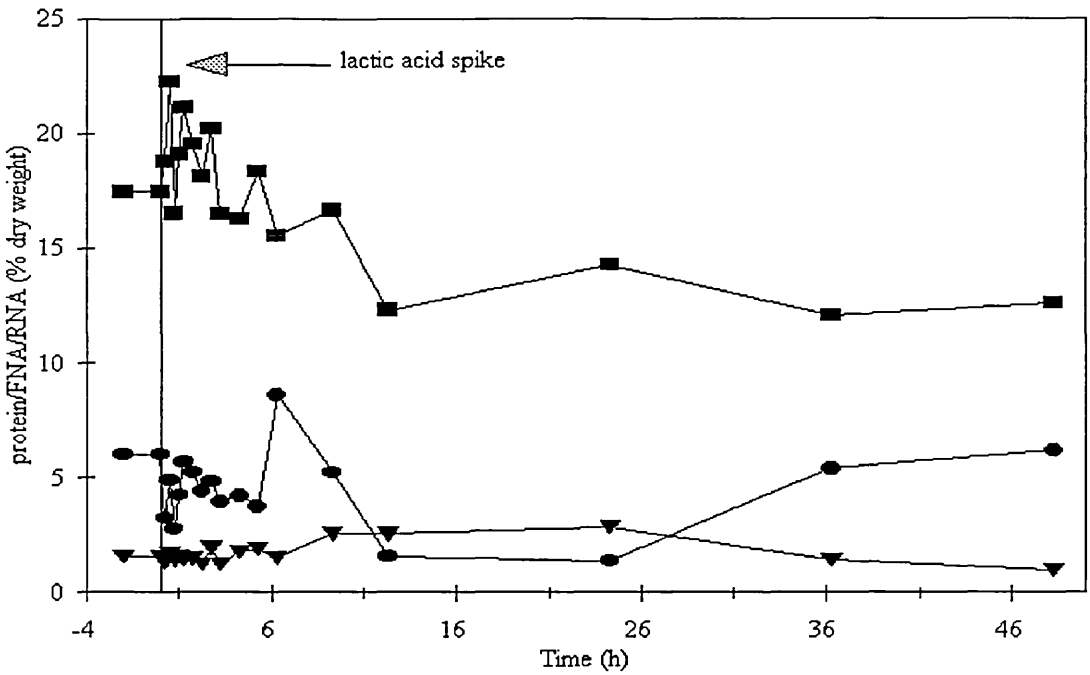
b) supra-optimal growth rate, $0.22h^{-1}$



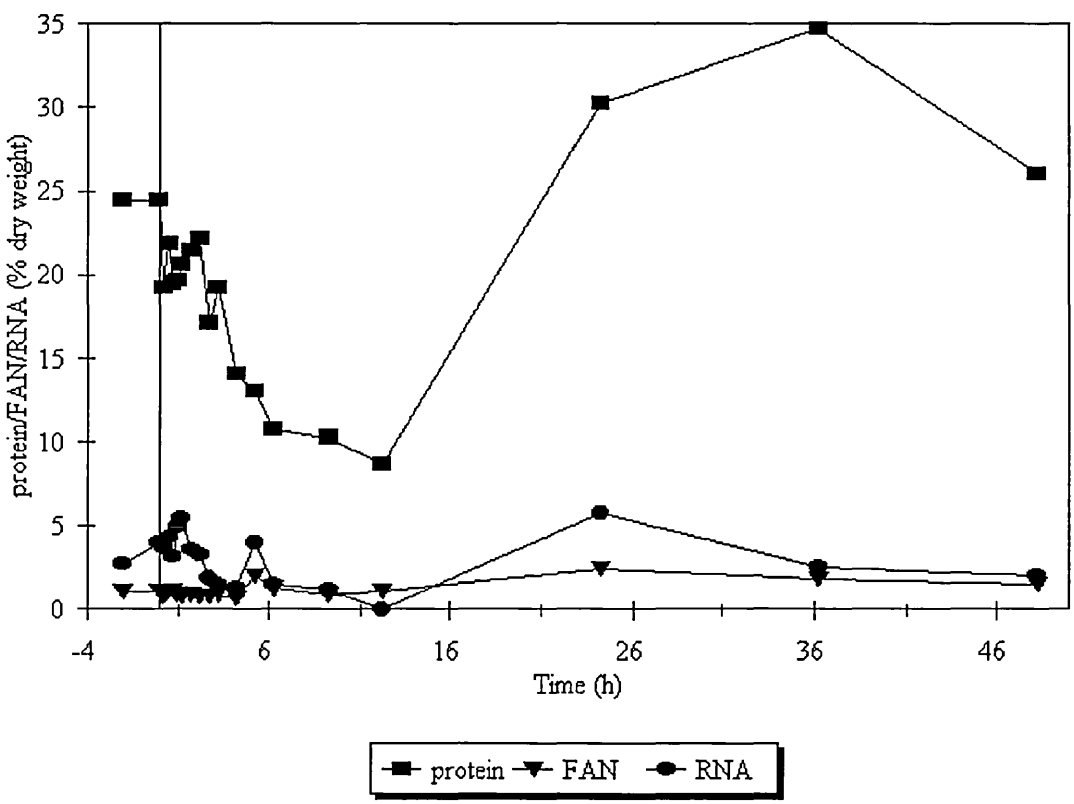
In Figure 6.2 the effect of the lactic acid spike was studied on the main nitrogen containing fractions of the yeast. The major effect seemed to be on the levels of cell protein, with little effect on RNA and FAN. All three of these factors oscillated for the first 6 hours before settling down at more steady levels for the duration of the samples with the protein being significantly down on steady state levels. The overall levels of protein reduced from ~17.5% to ~12.5%. It was assumed that the levels would return to steady state values as the biomass reached it's steady state value (protein was not determined beyond 48hr).

Figure 6.2 Effect of a lactic acid spike on yeast protein, FAN, and RNA. Baker's yeast was grown continuously under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



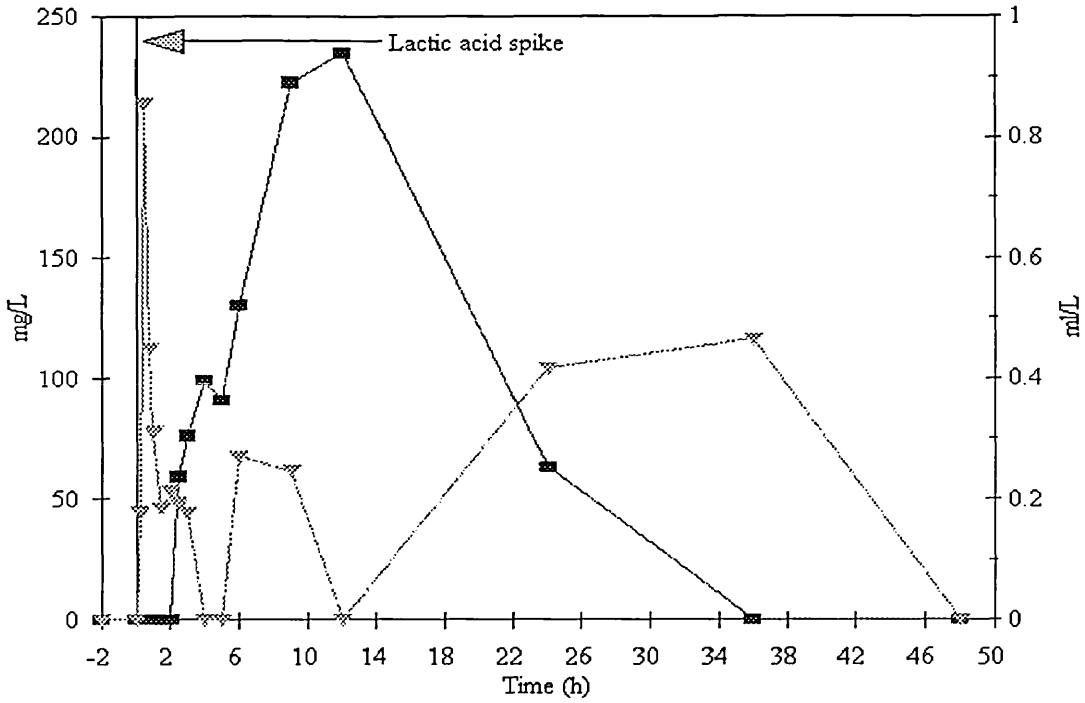
b) Supra-optimal growth rate, $0.22h^{-1}$



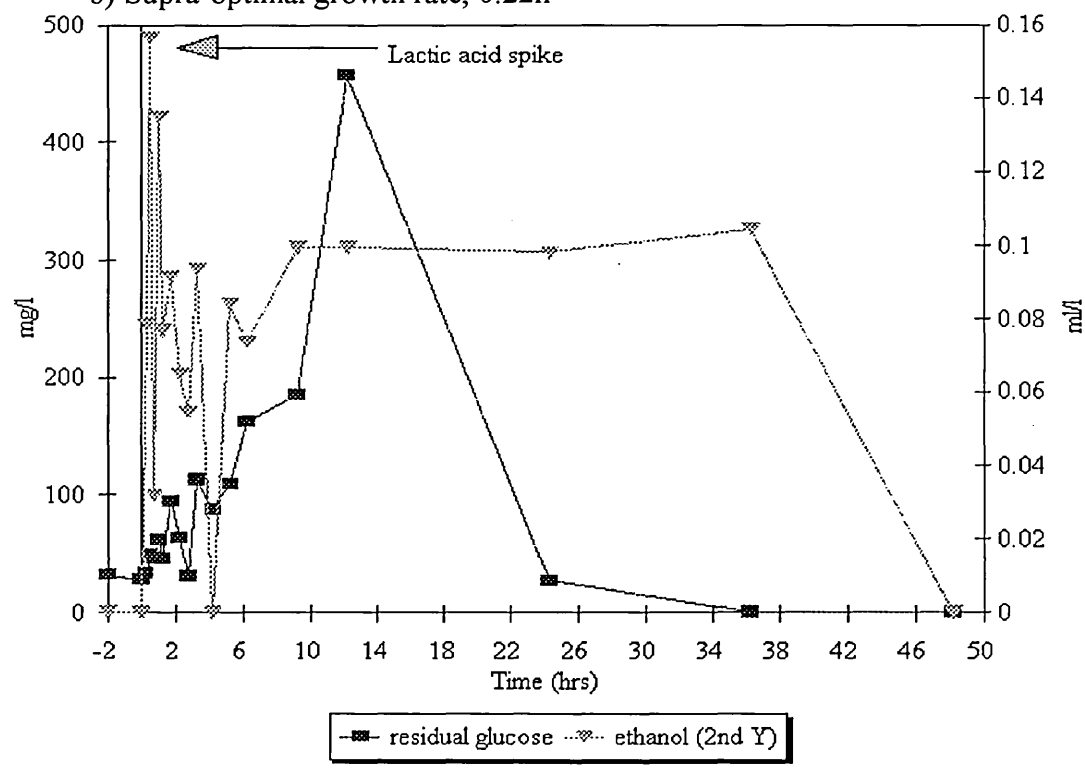
Figures 6.3 and 6.4 show what was happening in the extracellular environment during the lactic acid spike. Most notably from Figure 6.3b and 6.4b it can be seen that the yeast cells actually appear to take up the lactic acid quite quickly after the first 6 hours as compared to the levels that should be present if the lactic acid is only removed form the chemostat through physical washout. These graphs also showed that considerable amounts of succinate were released from the cells at the time of the acid spike. This is consistent with a shift or change of metabolism (Franco *et al.* 1985) which requires less throughput from the TCA cycle. In the super-optimally grown yeast, the levels of succinate released from the cells was less than that excreted from the sub-optimally cultured cells. Significant amounts of citrate were also released, above that normally found in the medium (results not shown). The other notable effect is shown in Figure 6.3a and 6.4a where both residual glucose and ethanol levels rose through the time of this spike. Ethanol rose quite markedly in the first 2h before being consumed by 6h in the sub-optimally cultured cells. This was then followed by another production/consumption cycle that finished after 12h. Levels of ethanol then appeared to remain relatively high until 48h when it was completely removed. In the case of the supra-optimally cultured cells, much less ethanol was produced from these cells although there was a long period (12-36h) where the level appeared to remain unchanged, but again ethanol was completely removed by 48h.

Figure 6.3 Effect of a lactic acid spike on yeast glucose metabolism. Baker's yeast was grown under glucose-limitation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



b) Supra-optimal growth rate, $0.22h^{-1}$



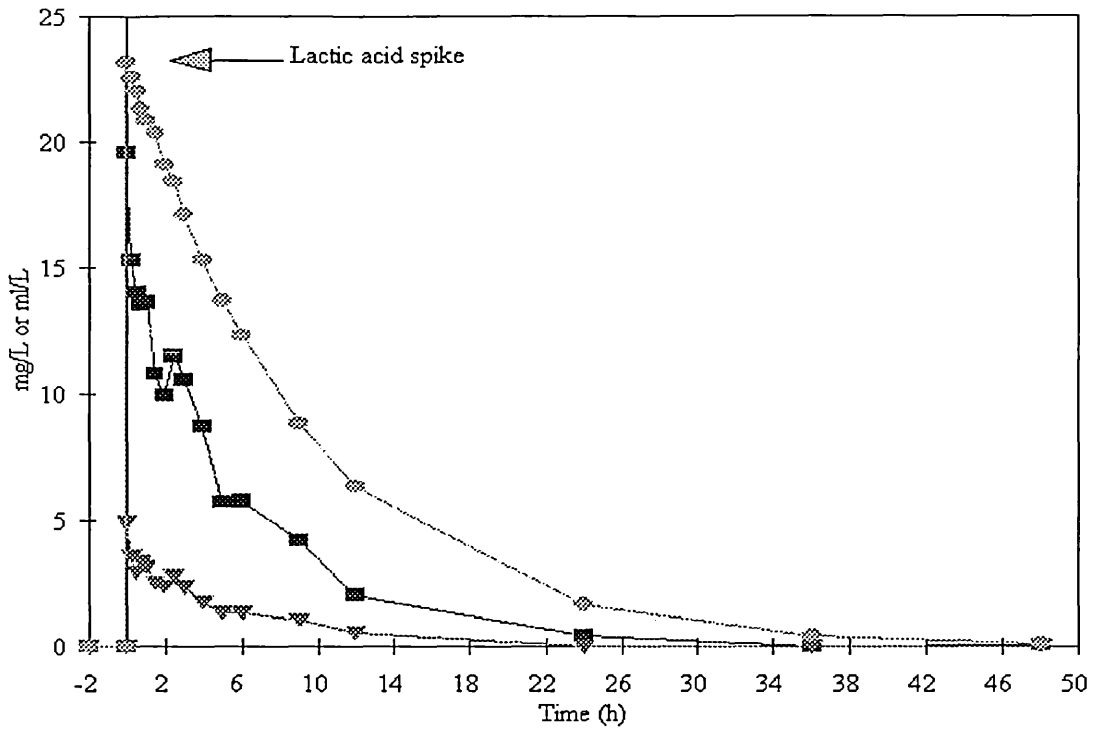
Throughout this oscillating ethanol production period the residual level of glucose in the medium rose significantly at 12h, with almost 25% of glucose added still remaining in the culture medium compared with 0% during normal steady state conditions. The residual glucose levels were significantly reduced by the time biomass recovery occurred at ~24h.

It was thought that the changes in the metabolic rate of *S. cerevisiae* must be due to switching from the fully respiratory state to the respiro-fermentative state. This may have been due to a decrease in oxygen uptake by the cells which would explain the rapid production of ethanol observed in the early supernatant samples (within 30min). However, oxygen electrode studies of the respiratory yeast subjected to a 2%v/v lactic acid spike showed no change in respiratory function over a 10-15 minute period.

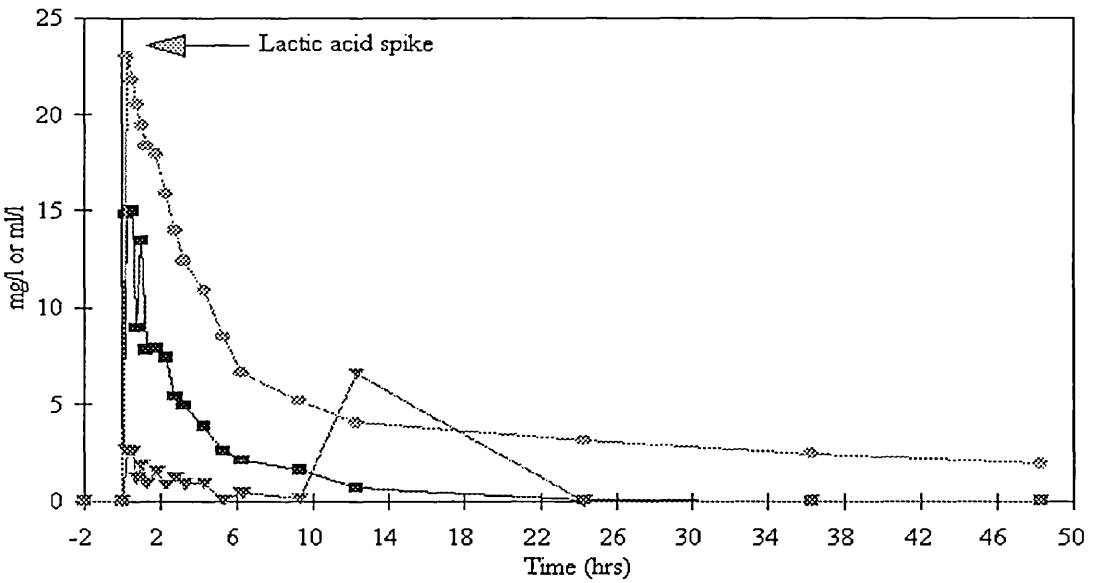
Research carried out by Jones (1968), suggested that yeast cells growing continuously in the presence of glucose took up to 24h to start utilising lactate as sole carbon source (Jones, personal communication). Jones (1968) also showed that the respiratory capacity (assessed by manometry) of yeast grown on glucose reduced as dilution rate increased, when supplied with lactate as a sole C-source.

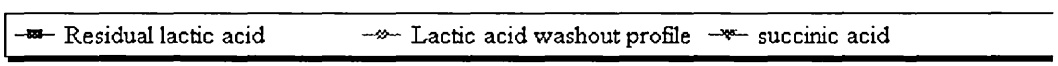
Figure 6.4 Effect of yeast on the rate of washout of a lactic acid spike from a chemostat. Baker's yeast was grown under glucose-limtation in a chemostat.

a) Sub-optimal growth rate, $0.12h^{-1}$



b) Supra-optimal growth rate, $0.22h^{-1}$





From the results obtained it is apparent that a 2% lactic acid perturbation caused the yeast cells to react at both a cell cycle and at a metabolic level. The cells clearly stop growing and reproducing, but it also appears that their predominant metabolic pathway changes, from a respiratory state to one which relies on respiro-fermentation. Alternatively, from the oxygen electrode studies performed in this research and by Jones (Jones 1968), it may be possible that the high level of lactic acid presented to the yeast cells may have passed into the cell in sufficient amounts to lower intracellular pH and alter the metabolic flux in favour of ethanol production without detrimentally effecting oxygen uptake. Transport of lactate and other short length organic acids by *S. cerevisiae* has been described (Leao and van Uden 1986), although in the case of lactate the transport in cells grown on glucose was shown to be by passive diffusion of the undissociated acid across the cell membrane. Diffusion across the cell membrane increases with increasing external pH (Leao and van Uden 1986) suggesting that the permeability of the membrane to undissociated lactate decreased linearly with increasing extracellular proton concentration. Leao and van Uden (1984) made similar observations in *Candida utilis*. This would suggest that in the current experiments the uptake of undissociated lactic acid would increase due to increased undissociated acid at the low pH conditions (pH 4.5-3.2 of QEMM3) during the experiment. However, diffusion across the membrane would be inhibited due to decreased permeability of the membrane to undissociated lactic acid at the same low pH.

Weak organic acids are used in the food industry as inhibitors of microbial growth due to their ability to preserve the food in a natural mechanism. However the

mode of action of weak acid preservatives, which includes lactic acid, involves extending lag phase and promoting cell stasis (Stratford and Anslow 1996) which may not be sufficient for preservation of foodstuffs. Recently it has been shown that this is a flawed approach as in most cases spoilage of food or beverage products occurs through fermentative action of the organism and not by cellular growth. This was particularly well exhibited in research which showed that for the yeasts *S. cerevisiae* and *Zygosaccharomyces bailii,* benzoic acid, acetic acid and propionic acid, used at normal preservative levels merely act to inhibit yeast growth but not yeast fermentative activity (Ferreira *et al.* 1997). Another problem with this approach is that some spoilage yeasts, especially *Zygosaccharomyces bailii*, are capable of metabolising the acetic acid even when glucose is present, resulting in the increased possibility of spoilage by other organisms as the yeast removes the preservative.

The results obtained in these experiments suggest that lactic acid behaves in a similar way to the other weak acids that are described in the literature. Certainly the change in pH that occurs in the culture is not believed to effect the cells directly. Some workers have shown that the toxicity of weak acids is dependent on the pH of the environment. In studies with acetic acid the lowest pH at which growth occurred with a strain of *S. cerevisiae* was 2.5. At pH 4.5, however, growth was eliminated in the presence of acetic acid (Taherzadeh *et al.* 1997). This was also shown to affect the cells at a metabolic level before preventing the growth of the cells, with a 20% rise in ethanol yield, while serving to reduce biomass yield by 45% and glycerol yield by 33%. These are all events which would be much more beneficial to industries relating to yeast fermentation rather than yeast biomass production. For biomass producers, the presence

of any weak acid in the medium may be detrimental to optimal production of yeast biomass, but as discussed above, the toxicity of an acid (like lactate) will depend on many factors, including environmental conditions and the physiological condition of the yeast.

CHAPTER 7 - CONCLUDING DISCUSSION

7.1. Physiology of baker's yeast under glucose-limiting conditions

In studying the yeast strain *S. cerevisiae* GB4918 in continuous and batch culture, it has been possible to determine some important physiological characteristics of this industrial microorganism. For example, during growth in varying levels of glucose in a defined medium it was possible to show that 1g/l glucose (0.1% w/v) was low enough to allow cellular respiration in the absence of fermentation. Occasionally, it was shown that incomplete aeration could result in fermentation of 1g/l glucose. However, in chemostat experiments no ethanol was detected and biomass yields were close to 0.35g biomass per g glucose consumed (gB/gG), close to the theoretical maximum of 0.5gB/gG that has been obtained for aerobically propagated *S. cerevisiae* (Weusthuis *et al.* 1994a). The expected yield for biomass in an anaerobic propagation (fermentation) is normally around 0.1gB/gG (Weusthuis *et al.* 1994a).

The main reasons for such a discrepancy in biomass yield could be the use of glucose for the production of metabolites, amino acids and other cellular components using additional nutrient sources in the medium, especially nitrogen and sulphur. These two elements are provided in a form that yeasts are able to readily utilise; nitrogen as ammonium, and sulphur as sulphate. Both of these are essential in the production of amino acids which are required in protein biosynthesis. The utilisation of both ammonium and sulphate require the expenditure of cellular energy to provide amino acids. A more accurate biomass yield may be obtained by taking into account these other

nutrients in calculating a mass balance, and this would require more intensive analysis of the spent medium and exhaust gases in order to establish the cellular fate of all the nutrients.

The only intracellular compound that was not affected by changes in growth rate was the free amino nitrogen content of cell digests. This confirmed that the cell culture was growing well and that cellular metabolism of the cell was, in general, balanced with respect to amino acid synthesis and utilisation. The levels of protein, trehalose, glycogen and RNA did, however alter with growth rate. Trehalose and glycogen were accumulated in greater amounts in cells growing at slow growth rates or in those cells whose growth rates were close to μ_{max}, where the onset of respiro-fermentative metabolism occurred. Results obtained from chemostats placed μ_{max} at around $0.24h^{-1}$, a result that is consistent with the range reported for the maximal growth rate in *S. cerevisiae* at which respiration is the predominant form of metabolism (Van Hoek *et al.* 1998b). The trends seen in protein, RNA, trehalose and glycogen in this work with cells grown in glucose-limiting conditions were similar to those reported by Ertugay and Hamamci (1997) for *S. cerevisiae* growing with sucrose as carbon and energy source. At 3g/l sucrose, the cells may preferentially ferment, although experiments of Ertugay and Hamamci (1997) describe aerobic conditions. However, there are inconsistencies in these experiments, for example, there is a high residual glucose level of 0.2g/l, and an unexplained biomass yield. The use of yeast extract in the medium used by Ertugay and Hamamci (1997) may have introduced extra glucose sources. The result of growing cells under the conditions described above is that fermentation may be an unavoidable metabolic use of sucrose and therefore the chemostat will be able to continue growth

beyond wash-out for an aerobic culture. The predominance of fermentative metabolism also means that cells exhibit different characteristics to that shown by the aerobically grown yeast studied in this thesis. With regard to the behaviour of trehalose, this disaccharide is known to be accumulated to greater extents in cells that are faced with some form of physiological stress. This often means that cells fermenting glucose, or other sugar sources, will have a greater effect on the activity of the trehalose synthesis and breakdown process, due to the combined effects of glucose repression on the synthetic enzymes, and the stimulatory effect of acetate and ethanol production. There is also support for trehalose metabolism being responsible for some control over the glycolytic pathway (Thevelein and Hohmann 1995). The regulation applied by trehalose synthesis on the glycolytic pathway could be lessened in conditions where glucose utilisation was limited by the lack of glucose, as in this study. These prevailing conditions of C-limitation may also confer a small physiological stress on the cells that results in a reasonable level of trehalose accumulation at low dilution rates. Certainly, accumulation of trehalose at low yeast growth rates is consistent with sugar-non-limiting conditions (Ertugay and Hamamci 1997).

Increased levels of protein and RNA in cells as growth rates increased was probably due to increased cell activity as the cells adapted to faster rates of nutrient feed. This could involve increased glycolytic flux through the possible increased synthesis or activity of glycolytic enzymes and increased expression of electron transport factors, such as the cytochromes. Transport proteins may also become more numerous as the cells attempt to translocate as much of the nutrients being supplied to them as possible. Measurement of other residual nutrient components would have been necessary to

identify the more efficient use of ammonium, phosphate, etc. It was certainly shown that as cells change to respiro-fermentative metabolism, biomass levels fell. During this time it was also recorded that the residual glucose concentration increased, probably due to the loss of biomass.

7.2 Physiology of the Crabtree effect in yeasts

It was shown in establishing glucose-limitation that the cells were responding to glucose availability by altering cell volume. The effect of ethanol, a respiratory C-source, was studied but showed no change in cell volume. It was of interest to establish physiological responses to glucose that may be part of the Crabtree effect. In comparing the baker's yeast strain of *S. cerevisiae* GB4918 (Crabtree positive) with a Crabtree negative *K. marxianus* strain it was possible to identify differences in Crabtree-related cell physiology. Certainly changes in cell size were identified as a major difference between the cells. The 50% increase in cell size in *K. marxianus* was quite dramatic, and this would provide the cell with a greater surface area to allow oxygen transfer, and glucose uptake, either passively or facultatively. However, the change in the size of *S .cerevisiae* was even more dramatic. While this was also accompanied by greater glucose uptake, oxygen uptake was rapidly reduced. This provided evidence of the utilisation of glucose by fermentative metabolism, confirmed by the production of ethanol, and greater amounts of glycerol production, which is essential in fermenting cells, for the regeneration of cytosolic NAD^+, necessary for continued operation of glycolysis. Ethanol was eventually produced in the *K. marxianus* strain but this may have been due to overflow metabolism at the level of mitochondrial pyruvate dehydrogenase (Pdh) or possibly due to reduced oxygen transfer. Some reports have

suggested that the activity of the pyruvate dehydrogenase bypass acts during aerobic growth of *S. cerevisiae* and that when the enzymes of the bypass become saturated ethanolic fermentation begins, especially in continuous culture when μ_{max} is passed (Van Hoek *et al.* 1998b). It has been shown, however, that the activity of acetyl CoA synthetase is not responsible for the build-up of acetic acid from yeast (de Jong-Gubbels 1998), suggesting that there must be a limiting step downstream of acetyl CoA, perhaps in the TCA cycle itself.

There was little effect on the protein content of either yeast species studied but the change in RNA concentration was probably due to increased growth of the cells. As described earlier, trehalose and glycogen synthesis were both repressed by glucose and accumulation of trehalose tended to occur under stressed conditions while glycogen accumulated as glucose became exhausted. The reactions of both *S. cerevisiae* and *K. marxianus* was consistent with this behaviour. As the available glucose increased, both yeasts reduced glycogen and trehalose content until the glucose feed returned to normal, and excessive levels of glucose had been removed from the chemostat.

7.3. Perturbation of yeast physiology

7.3.1. Cations

The effects of zinc and calcium perturbations in chemostat cultures were, to some extent, dependent on growth rate. The main effect of zinc and calcium perturbations on the yeast in chemostat cultures was on trehalose and glycogen metabolism. No effect was noted on overall biosynthetic metabolism and there were minimal effects on protein biosynthesis by calcium. Clearly, the major effect of calcium was to reduce overall levels

of trehalose and glycogen while increasing protein present in the cells. The major reason for the reduction in trehalose levels was almost certainly due to the activity of the catabolic enzyme, cytosolic-trehalase (c-trehalase), which has a requirement for calcium or manganese (Thevelein 1996) and is inhibited by magnesium and zinc (Salek and Arnold 1995). The addition of extra calcium may lead to an increase in the rate of degradation of trehalose. Control of glycogen metabolism is much more complex than that for trehalose. Certainly, the stages that produce the building blocks of glycogen require magnesium as a co-factor and are presumably inhibited by calcium. This includes the phosphoglucomutase reaction and the adenyl-, uridyl-trasnferases that produce ADP- and UDP-glucose. The involvement of UDP-glucose in the production of both trehalose and glycogen is well known but the involvement of ADP-glucose is less well described. The original belief of an alternative trehalose pathway that was induced by maltose was dismissed by Petit and Francois (1994) as being due to the ineffective repression of the trehalose synthesis system . The existence of the ADP-glucose linked system comes from evidence that Δ*tps1* (trehalose 6-phosphate synthase) mutants, which did not accumulate trehalose, were able to sporulate, a process that requires trehalose (Ferreira and Panek 1993). Results presented in this work also support the existence of a secondary trehalose accumulation process. This process was not stimulated by heat shock, but appeared to be enhanced by increased magnesium availability. As explained earlier, the enzymes involved in the known UDP-glucose-dependent trehalose synthase complex have an absolute requirement for magnesium. The same may also be true for an ADP-glucose dependent trehalose synthase complex.

Both calcium and zinc appeared to increase the levels of protein present in the cells and this may be due to increased expression of protein that are used to transfer and store these cations. Both are required in very small (micromolar) quantities but both can also be inhibitory towards yeast growth. The protein calmodulin is believed to act as a "calcium mop" for excess calcium that is not required for the few metabolic reactions that it takes part in (e.g. lipid metabolism). Calcium appears to be treated as more of a hindrance to the cell and can be actively excluded (Walker *et al.* 1996). This is in contrast to zinc which is known to be essential in protein stabilisation and as an enzyme co-factor. Excess zinc is stored in the vacuole (Dedyukhina *et al.* 1989), as is involved in differential protein biosynthesis by yeast (Obata *et al.* 1996).

The utilisation of these perturbed cells in yeast applications such as baking and brewing may lead to the identification of favourable or unfavourable changes to yeast performance. Specifically, the addition of extra zinc to brewery fermentations is known to stimulate "stuck" fermentations and enhance the performance of yeast fermentation in general (Bromberg *et al.* 1997); (Rees and Stewart 1998).

7.3.2. Organic effectors

In the perturbation of steady state cultures with 2% (v/v) lactic acid there were dramatic changes to the cell most of which may be due to altered yeast metabolism. If the cells were truly in a de-repressed state, with regard to glucose, then lactic acid utilisation should have occurred relatively quickly. Previous research has suggested up to 24h adaptation to lactic acid by *S. cerevisiae* (Jones 1968). However, the lack of

correlation between chemostat washout profile of lactic acid, and measured washout of lactic acid showed that the cells were apparently taking up some of the lactic acid. It is also likely that while the action of the lactic acid was significant, it is not thought that the intracellular acidification of the cytosol is the reason for the changes. Lactic acid has been shown to inhibit yeast growth at concentrations around 800mM and it also exhibits a poor chelation ability, unlike citric acid or EDTA (Stratford 1999). In fact, the drop in extracellular pH caused by a lactic acid spike was only of the order 0.8, from 3.2 to 2.4. This final pH value was quickly raised by the yeast back to 3.2 (24h) and lowered to 2.4 during the Crabtree-related experiments. The effect of lactic acid perturbations were also dependent on growth rate of the cells. The most dramatic effects on trehalose and glycogen were evident in cultures grown at the sub-optimal growth rate, while the greatest effect on protein occurred in the supra-optimal growth rate culture. The loss of biomass was consistent at both growth rates, and the effect on yeast biomass levels was probably due to washout of cells adapting to utilisation of lactic acid and reduced biomass yield from the cells growing on lactic acid. It would appear that at the lower growth rate, increased levels of glycogen and trehalose are indicative of a survival response to the lactic acid perturbation. Once biomass began to recover it was noticeable that both these carbohydrates levels were reduced, probably due to the dilution effect of increased growth.

7.4. Critique

The difficulty in reproducing these experiments may be due to unavoidable variations in culture handling, especially in pre-culturing for use in the chemostat. Care must be taken to ensure a uniformity of culture handling, especially the use of cells in a

physiologically defined state. The results of the chemostat experiments presented in this thesis represent the trend that occurs when yeast cells are challenged or perturbed and in each case, quantitation may not be absolute. This is a fault of the experiment set-up, where in each case, the logistical exercise lead to duplicate experiments being carried out on different cultures. Contamination of chemostats after one particular perturbation lead to duplicates being carried out on entirely new chemostat cultures. This is particularly relevant to perturbations involving cationic perturbations, where the yeast cells may store excess calcium or zinc (e.g. in vacuoles). This would have masked the effect of another spike of the same, or a different effector, had these experiments been carried out on "recovered" cultures. The use of such "recovered" cultures may have been interesting in terms of studying another effector known to counter the effects of the first, e.g. a magnesium spike to see if trehalose accumulation returned to pre-calcium spike levels. This would involve longer exposure of cells to calcium increases, by the using a "pulse" of calcium that means continuing the growth of the yeast in elevated levels of calcium. The physiological characteristics of cells grown in these conditions could be assessed and described and then exposed to a spike of magnesium, and study resultant changes in cell physiology characterised.

The use of "recovered" cultures previously exposed to effectors such as lactic acid was deemed practical, and it may be possible to "condition" the yeast to the exposure of 2% lactic acid. This was not determined in this thesis, although the response to duplicate lactate spikes was definitive. For example, at each dilution rate, the magnitude of the second spike was smaller, suggestive of conditioning. Further investigation into 3rd and perhaps 4th spikes with lactic acid may help discern if the yeast

cells were being conditioned to the addition of lactate. It would also be of use to study the effect of other organic effectors that may be present in different molasses stocks (e.g. fatty acids, aldehydes, pesticide residues etc.). Further analysis of molasses, perhaps by fractionation, may have allowed the identification of particular components that were more beneficial/detrimental to yeast growth or fermentation.

The results presented in this thesis show that nutrient availability had a major influence on the macromolecular composition of *S. cerevisae*. This is of direct relevance to the production of yeast biomass for baking, brewing and yeast extracting purposes.

The influence of calcium on trehalose and glycogen implied that yeast cells may have a poorer resistance to stress conditions. This includes the conditions used for autolysis and suggests that raised calcium levels may aid in increasing the rate of yeast autolysis. Interestingly, in mammalian cells, increased levels of intracellular calcium are known to accelerate cell death (Walker, 1999). The increases seen in protein levels during zinc spikes suggest that this may be beneficial in producing a yeast with a better protein content for food yeast or yeast extract manufacturers. In conjunction with raised calcium levels it may be possible to produce high protein content cells which may be easier to extract. The zinc-enriched yeast may also be beneficial to bread and brewing industries, as the zinc would allow for a more effective fermentation performance (Rees and Stewart, 1998).

When *S. cerevisae* is subjected to molasses feedstocks that are contaminated with organic acids cells would be severly effected, with regard to biomass production,

depending on the growth rate of the yeast and the amount of organic acid present. The results presented here are for lactate, which has been shown to be less effective at inhibiting yeast growth than many other organic acids (such as benzoic, acetic, sorbic Stratford and Anslow, 1996). This identifies the important need for suppliers or consumers of molasses feedstocks to analyse molasses for more than simply sugar content.

CHAPTER 8 - REFERENCES

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